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In-vitro Propagation and Fish Assessments to Inform Restoration of Dwarf Wedgemussel (Alasmidonta Heterodon)

Jennifer Ryan

IN-VITRO PROPAGATION AND FISH ASSESSMENTS TO INFORM RESTORATION OF DWARF WEDGEMUSSEL (*ALASMIDONTA HETERODON*)

A Thesis Presented

by

JENNIFER ELAINE RYAN

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Approved as to style and content by:

_____
Allison H. Roy, Chair

_____
David L. Perkins, Member

_____
Peter D. Hazelton, Member

_____
Curtice R. Griffin
Environmental Conservation

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ABSTRACT

PROPAGATION AND FISH ASSESSMENTS TO INFORM RESTORATION OF DWARF WEDGEMUSSEL (*ALASMIDONTA HETERODON*)

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JENNIFER E. RYAN, B.S., STATE UNIVERSITY OF NEW YORK AT POSTDAM

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Allison H. Roy

The dwarf wedgemussel (*Alasmidonta heterodon*) is a federally endangered freshwater mussel that once ranged from New Brunswick to North Carolina, but now only exists in isolated populations throughout its diminished range. Laboratory propagation in conjunction with augmentation or reintroduction is considered a critical component of dwarf wedgemussel restoration. My thesis aimed to I) develop *in-vitro* propagation techniques including methods to minimize microbial contamination for two *Alasmidonta* species and II) compile and assess critical fish assemblage information at existing dwarf wedgemussel presence and absence locations to inform future restoration activities. For the first objective (Chapter 2) I assessed three methods of contamination mitigation (media change frequency, concentration of the antifungal Amphotericin B, and method of antifungal replenishment). Across all experiments, higher levels of contamination severity had a negative impact on transformation success. In my experiments, dishes that had media changes every other day (vs. those changed daily and every 3 days) had the highest contamination and the lowest glochidia transformation success. Treatments with

the lowest (0 µg/mL) concentration of the antifungal, Amphotericin B, and got a low-dose replenishment of Amphotericin B (vs frequent media changes) had the highest transformation success. The fungus was identified as *Candida parapsilosis*, a common fungus found in aquatic and human environments; future propagation efforts should use mitigation methods that are specific to the fungal contaminant. In the assessment of host fish near dwarf wedgemussel locations (Chapter 3), two fish repeatedly emerged as indicators of dwarf wedgemussel occurrence and abundance: the tessellated darter (*Etheostoma olmstedi*) and brown trout (*Salmo trutta*). Tessellated darter was positively related to dwarf wedgemussel which supports the importance of tessellated darter as a host fish in the wild. The negative relationship between dwarf wedgemussel and brown trout may indicate a difference in habitat between the species or brown trout's predation upon wild hosts. Results from this project will be used in conjunction with habitat and genetic information to inform future restoration plans in the northeast and add to the growing body of literature on *in-vitro* propagation of freshwater mussels.

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CHAPTER 1
INTRODUCTION

1.1 Study Species – The Dwarf Wedgemussel (*Alasmidonta heterodon*)

Freshwater mussels (Family Unionidae) are the most imperiled group of organisms in North America (Haag 2013). Sixty-five percent of remaining mussel species are threatened, endangered or vulnerable (Haag and Williams 2013). The most imperiled group among them are members of the *Alasmidonta* genus. Of the 13 belonging to this genus, 3 are extinct (Bogan 2000; Cummings and Cordeiro 2011) and 8 are considered endangered, threatened, or a species of concern (Williams 1993). One member of this genus, the dwarf wedgemussel (*Alasmidonta heterodon*), is the only federally listed species in the Northeastern United States (US Fish and Wildlife Service 2007). The dwarf wedgemussel once ranged from the Neuse River in North Carolina to the Petitcodiac River in New Brunswick, Canada but its range has since diminished (Michaelson 1995), and in 1990, the dwarf wedgemussel was placed on the U.S. federally endangered species list (USFWS 2007). The dwarf wedgemussel, like other imperiled freshwater mussel species, have experienced declines due to overharvesting in the late 1800s (Pritchard 2001), stream impoundments, channel alterations, pollution (Campbell and Hilderbrand 2016), as well as causeway construction (Locke 2003). Dwarf wedgemussels have also likely declined due to their complicated life history, which relies on a host fish, and their predominantly sedentary nature, which makes it difficult for the mussel to escape disturbances (Galbraith *et al.* 2020). Currently, there are fewer than 30 healthy remaining populations of dwarf wedgemussel (Galbraith *et al.* 2020).

In the northeastern U.S., the dwarf wedgemussel persists in 15 viable populations across 2 watersheds: the Connecticut River and the Delaware River watersheds (USFWS 2019). While dwarf wedgemussels in the Connecticut River watershed remain largely understudied, populations in the Delaware River watershed have been heavily studied for their habitat (Strayer and Ralley 1993, Maloney *et al.* 2012) and flow preferences (Parasiewicz *et al.* 2017) and thermal tolerances (Galbraith *et al.* 2020). In the Maryland Coastal Plain, species distribution modeling revealed that dwarf wedgemussel is associated with low elevation streams, deeper depth to groundwater, low to moderate levels of Tertiary-aged deposits and little development, and agriculture or woody wetlands (Campbell and Hilderbrand 2016). Low intensity development in catchments resulted in the most positive predictor variable for dwarf wedgemussel occurrence (Campbell and Hilderbrand 2016), which may indicate that the dwarf wedgemussel is a highly sensitive species with low tolerance for pollutants. Other studies have found that dwarf wedgemussels tend to persist in and move toward sandy substrates (Strayer and Ralley 1993) but are absent from areas in streams that are highly silted (Parasiewicz *et al.* 2017). Their absence from highly silted areas may be caused by lower oxygen availability for juvenile mussels by clogging interstices in stream where juveniles attempt to mature following excystment from their hosts (Parasiewicz *et al.* 2017). Additionally, mussels that were found in the main channel of the Delaware River were associated with areas with low velocity relative to the whole main channel (Maloney *et al.* 2012). Shallow areas of streams with stagnant water may require a steady influx of cold groundwater for dwarf wedgemussels to persist (Briggs *et al.* 2013). This idea is further supported by research indicating that dwarf wedgemussels are sensitive to high temperatures; dwarf

wedgemussel can survive in temperatures up to 32°C to 36°C, but in an experimental setup, tended to move toward 16°C (Galbraith *et al.* 2020). When describing potential dwarf wedgemussel habitat, it is important to keep in mind that dwarf wedgemussel, like many rare species, are not always found in suitable habitats and may sometimes be found in sites that are largely unsuitable for the species. This makes it difficult to know true habitat preferences for the dwarf wedgemussel, as the habits where mussels are found may simply be artifacts of previously suitable habitat (Campbell and Hilderbrand 2016). Additionally, dispersal limits to the dwarf wedgemussel may also be due to limits of the host fish rather than habitat (Schwalb *et al.* 2015).

Like all unionids, the dwarf wedgemussel has a unique reproductive life cycle that requires host fish to successfully reproduce (McLain and Ross 2005, Lima 2012). In late summer and early fall, male mussels release their sperm into the water column, which are then filtered by sexually mature female mussels to produce larvae called glochidia (Haag 2012). The glochidia develop and are stored in the gills of female mussels throughout the fall and winter, a reproductive strategy known as long term brooding (Haag 2012). The dwarf wedgemussel, compared to other freshwater mussel species, has relatively low fecundity, only producing approximately 2,500 glochidia per reproductive year (Haag 2013). Dwarf wedgemussel release their glochidia into the water column from April to mid-June when temperatures reach 10–20°C (i.e., earliest are in the southern part of their range); then glochidia attach themselves onto the gills and body of a host fish (McLain and Ross 2005). Although the dwarf wedgemussel is considered a host generalist, meaning it can transform on host fish from different families (St. John White 2007, Haag 2012), dwarf wedgemussel glochidia have only been identified and observed on the gills

of tessellated darter (McLain and Ross 2005). The host fish provide nutrients to the glochidia and serve as the primary means for mussel dispersal (Strayer 2008). After two to three weeks, glochidia drop off fish as transformed juveniles and settle into the sediment (Michaelson and Neves 1995). More than 99% of the glochidia developed by the gravid female mussel never successfully develop into juveniles due to a combination of low attachment and transformation rates on fish (Lima 2012). The mussel's survival and future reproduction after dropping off their host fish depends primarily on securing suitable habitat and the availability and quality of food, which consists of particulates less than 20 µm, including phytoplankton and dissolved organic matter (Haag 2012). The age at which the dwarf wedgemussel becomes sexually mature has not been reported; however, once mature, the species is thought to reproduce annually until it dies. The dwarf wedgemussel may live to be as old as 12, however, the oldest individual found and recorded was only 9 years old (Michaelson and Neves 1995).

1.2 Freshwater Mussel Propagation and Host Fish for Restoration

Since 1899, propagation and reintroduction have been used to restore freshwater mussel populations that drastically declined due to overharvesting for their pearls (Patterson 2018) and shells for button making (Anthony and Downing 2001). Beginning in the early 20th century, fish were infected with glochidia and released into streams and rivers at the Fairport Biological Station (Fairport, Iowa) in an attempt to restore the failed mussel beds (Pritchard 2001). Unfortunately, this program ended when scientists were unable to assess the program's accomplishments (Haag 2012) and financial support for mussel research ended (Pritchard 2001). In the 1990s, researchers started producing

freshwater mussels by infecting fish with glochidia (Haag 2012) then culturing juvenile mussels in the lab before releasing into the wild (Patterson 2018). This approach, called *in-vivo* propagation mimics the naturally occurring process where glochidia attach onto the fins and gills of a host fish and transform into juveniles (Haag 2012). Dwarf wedgemussel have been successfully propagated using *in-vivo* methods with slimy sculpin, shield darter, and other fish species, with various levels of transformation success (St. John White *et al.* 2017).

Although most freshwater mussel propagation efforts have used *in-vivo* methods, *in-vitro* propagation, where juvenile mussels are produced without a host fish, is another approach to propagation. *In-vitro* propagation often uses glochidia to their more efficient potential, obtaining higher transformation rates and maximizing reproductive success compared to *in-vivo* propagation (Lima 2012). The *in-vitro* method of propagation was first developed in the 1920s (Ellis and Ellis 1926); however, the ingredients used in this process were never reported and so methods could not be replicated (Lima 2012). It was not until over 50 years later in the 1980s when *in-vitro* propagation methods were revisited (Isom and Hudson 1982). Over the last 40 years, *in-vitro* methodology has been refined by numerous researchers (Keller and Zam 1990, Uthaiwan 2001, Owen *et al.* 2010) for dozens of species (Lima 2012). Current lab practices for *in-vitro* propagation transform glochidia via a media solution comprised of amino acids, lipids, antibiotics, serum, and other nutrients (Owen 2009). Media solutions developed for *in-vitro* culture vary in terms of amount and type of serum, antifungals, and antibiotics used. Transformation success during *in-vitro* propagation is affected not only by media ingredients, but also by temperature, CO_2 levels, and contamination severity (Owen *et al.*

2010). Although several species of freshwater mussels have successfully transformed using refined *in-vitro* methods (Owen 2009), with the exception of current research in North Carolina (Michael Walter, personal communication), there has been no *in-vitro* propagation of the dwarf wedgemussel.

Developing methods for propagation in the lab is only one step toward restoration. Before releasing propagated mussels into native habitats, it is important to ensure that the abiotic and biotic environment are sufficient for long term survival (Strayer *et al.* 2019). One critical aspect of the biotic environment is having an adequate number of host fish available for reproduction (Strayer *et al.* 2019). Researchers have long made the link between freshwater mussels and their host fish, suggesting that the local diversity of mussel species may be constrained by the number of available host fish (Vaughn and Taylor 2000). An understanding of fish populations near healthy dwarf wedgemussel sites and at potential augmentation sites is needed before designing a recovery plan involving reintroduction of mussels into the wild.

1.3 Study Objectives

In my research I used a combination of lab experiments and field observational data to collect critical information for restoring dwarf wedgemussel through propagation and augmentation/reintroduction. The first chapter of this thesis examined how microbial contamination severity and methods of mitigation affected transformation success *in-vitro* for two *Alasmidonta* species: dwarf wedgemussel and a surrogate species, triangle floater (*Alasmidonta undulata*). Contamination can be detrimental to glochidial development and drastically decreases transformation success (Owen *et al.* 2010). Over

two experiments, I investigated how frequency of media changes; concentration of an antifungal, Amphotericin B; and method of replenishment (via media changeout or supplemental dosing) influenced contamination severity and transformation success. By investigating these objectives, I could determine how to best mitigate contamination, potentially transforming a higher proportion of juveniles than with *in-vivo* propagation and better utilizing the glochidia of this low fecundity species.

The second chapter of my thesis assessed the relationship between fish abundance and dwarf wedgemussel occurrence and abundance in the Connecticut and Delaware River watersheds. Specifically, I characterized fish assemblages in both watersheds as they related to dwarf wedgemussel occurrence. I also studied the relationship between the abundance of potential host fish, as identified by St. John White *et al.* (2017), and how they related to dwarf wedgemussel abundance and occurrence. By learning more about the fishes near dwarf wedgemussel sites, we can better select augmentation and reintroduction locations where dwarf wedgemussels will have the opportunity to reproduce and ultimately persist long term. Results from this study—in conjunction with genetics, water quality, and habitat information— can help us better manage and preserve dwarf wedgemussel populations.

CHAPTER 2

ASSESSING METHODS FOR MITIGATING CONTAMINATION IN FRESHWATER MUSSEL IN-VITRO PROPAGATION

2.1 Introduction

Since the early 1900s, propagation and population augmentation or reintroduction have been used to restore freshwater mussel populations (Patterson *et al.* 2018). Historically, propagation mimicked natural reproduction, using host fish to transform glochidia to juveniles, a process called *in-vivo* propagation (Patterson *et al.* 2018). In the last 40 years, *in-vitro* methods have been developed to propagate freshwater mussels, where a media solution is used in lieu of a host fish. Effective media solutions developed for *in-vitro* culture can vary depending on the species and may consist of different amount and type(s) of serum(s), lipids, amino acids, antifungals, and antibiotics and vitamins (Owen 2009). *In-vitro* propagation often utilizes glochidia to a greater potential, obtaining higher transformation rates and more efficient use of the glochidia compared to *in-vivo* propagation (Lima *et al.* 2012). These higher juvenile outputs can be especially important for species with low fecundity.

Several species of freshwater mussels have been successfully propagated using *in-vitro* methods (Owen 2009; Lima *et al.* 2012), however, one key challenge to successful propagation is management of fungal contamination (Owen *et al.* 2010). Fungal contamination *in-vitro* causes damage to cells (Langdon 2010) and can kill glochidia before they transform (Monte McGregor, personal communication). Fungi are ubiquitous and live on our bodies and artificial materials, as well as in soil, water and air; however, depending on the amount and species, fungi can be harmful to organisms in the

environment (Trofa *et al.* 2008; Newbound *et al.* 2010). To combat fungal contamination in *in-vitro* propagation, researchers change the media frequently (as often as daily) (personal communication, Monte McGregor) to minimize detrimental effects to the glochidia. Additionally, the antifungal, Amphotericin B is added to the media to mitigate contamination (Owen *et al.* 2010). Amphotericin B is a polyene antibiotic that was the first commercially significant antifungal drug (Lemke *et al.* 2005; Gallis 1990) and has been widely used in a variety of cell cultures and to treat fungal infections in human patients (Steimback *et al.* 2017) in addition to propagation of freshwater mussels (Owen *et al.* 2010). Currently, Amphotericin B is administered as a comprehensive antifungal, as the species of fungi growing in culture is currently not known (Monte McGregor, personal communication). In the medical world, Amphotericin B is given in low doses to patients combating fungal infections because the drug is accompanied by dose-limited toxicities and can cause harm to the patient if given in high enough amounts (Hamill 2013). It is presumed that a similar harmful effect would cause low transformation in freshwater mussels, and thus many *in-vitro* formulas call for a low concentration of Amphotericin B (0.67 μg/mL – 5 μg/mL) to control fungal contamination (Monte McGregor, personal communication; Keller and Zam 1990). However, a 50 μg/mL dose of Amphotericin B did not detrimentally affect transformation of the cockscomb pearl mussel (*Cristaria plicata*), suggesting that glochidia of different freshwater mussel species may have varying tolerance levels for concentrations of Amphotericin B (Ma *et al.* 2018).

Contamination severity and Amphotericin B concentration may impact transformation success and are both important to understand for successful *in-vitro*

propagation. To date, no studies have quantified the effect of fungal contamination on mussel transformation or identified the species of fungi. The identification of the fungi is particularly important because it allows for specific treatment of the fungus (Ryan 1994) and broad spectrum antifungals, like Amphotericin B, often fail to mitigate target fungi (Leifert and Cassells 2001). The goal of this research was to determine how fungal contamination and methods of mitigation influenced transformation success of two *Alasmidonta* species, the dwarf wedgemussel (*Alasmidonta heterodon*) and the triangle floater (*Alasmidonta undulata*). My objectives were to 1) determine which methods of contamination mitigation—frequency of media change, concentrations of the antifungal Amphotericin B, and method for Amphotericin B replenishment—caused the least contamination severity, 2) identify the species of fungus in experimental dishes, and 3) assess how different methods mitigation affected mussel transformation success. This research will aid in the improvement of *in-vitro* propagation methods by developing ways to mitigate contamination while maximizing transformation for the propagation of freshwater mussels.

2.2 Methods

2.2.1 Study Species

The focus on this study was the dwarf wedgemussel (*Alasmidonta heterodon*), a small (< 45mm) mussel found in isolated, patchy distributions along the Atlantic slope from Massachusetts to North Carolina, where it is federally listed (USFWS 2007). The dwarf wedgemussel is considered a host generalist and has a maximum theoretical life expectancy of 12 years (Michaelson and Neves 1995).

The triangle floater (*Alasmidonta undulata*), a mussel belonging to the same family as dwarf wedgemussel, was used as a surrogate in one experiment to minimize the use of a federally endangered species. Today, triangle floater is listed in many states and considered vulnerable throughout much of its range (Cordeiro 2011). Both species are host generalists found throughout the Atlantic slope (Nedeau 2008). Triangle floater are slightly larger than dwarf wedgemussel and have higher fecundity (personal observation). Both species also share similar habitat preferences (Nedeau 2008).

2.2.2 Experimental Overview

There were 2 experiments completed in this study. Experiment 1 examined frequency of media changes and how different treatment levels (media change every 1 day, 2 days, and 3 days) impacted contamination severity and transformation success of dwarf wedgemussel glochidia. Experiment 2 examined and how different Amphotericin B concentrations (0, 1, 3, 5, and 10 µg/mL) impacted contamination severity and transformation success. Experiment 2 also compared replenishment methods for Amphotericin B— one treatment received frequent media changes while the other received a dose of Amphotericin B in place of a media change— and their impact on contamination severity and transformation success.

2.2.3 Broodstock Collection and Housing

For Experiment 1, two gravid dwarf wedgemussels were collected via view bucket and snorkeling by Biodrawversity, Inc. from the Mill River in Whately, Massachusetts in March 2019 and brought to the U.S. Fish and Wildlife Service's Cronin

Aquatic Resource Center (CARC) in Sunderland, MA. Mussels were housed in a flow through system with weekly water changes and held at 2ºC until day 0 of the experiment on June 13, 2020.

For Experiment 2, six gravid triangle floaters were collected from the Squannacook River in Townsend, Massachusetts in November 2019 using a combination of snorkeling and view buckets. Mussels were held in silos (adapted from Chris Barnhart; Missouri State University) in the river to mature until being used in experiments. The mussels were moved to CARC on January 2, 2020 where they were housed in a flow through system with daily water changes. The temperature was increased from 1.5ºC to 12ºC over 5 days for the start of the experiment on January 7, 2020. All mussels were measured, tagged, and cleaned before use.

2.2.4 Glochidia Testing and Media Preparation

Glochidia were extracted from individual water tubes from each mussel by rupturing one (for triangle floater) or both (for dwarf wedgemussel) gills with a water filled syringe with a 22G needle. Glochidia density and viability were tested using the salt test following Neves *et al.* (1985). Glochidia from the ruptured gills from each female were kept in separate beakers with 100 mL of sterile water. Five, 200-µl subsamples were taken from each beaker and 1 drop of salt solution (NaCl) was added to each subsample of glochidia. Glochidia that closed after the addition of salt indicated that the glochidia were viable and those that did not were considered not viable. Percent viability for each subsample was calculated using the following equation:

$$\left(\frac{Open\ before\ salt\ addition\ -\ Open\ after\ salt\ addition}{Open\ before\ salt\ addition\ +\ Closed\ before\ salt\ addition}\right) * 100$$

The mean of the 5 subsamples was calculated to determine viability for each mussel. Additionally, glochidia from the same 5 subsamples were counted and used to volumetrically estimate the number of viable glochidia in each gravid female.

After testing viability and volumetric estimates, glochidia from each mussel were combined into a single beaker filled with a filtered media solution (Table 2.1). The media was continuously agitated to prevent glochidia from snapping shut on one another. Debris from the mussel and non-viable glochidia were removed by repeatedly decanting the media off the top as the glochidia settled to the bottom. Additional debris and open glochidia were removed with a pipette.

For all experiments rabbit serum (33%) was added to the base media solution (67%) (Table 2.1). Prior to use, the rabbit serum was heat treated by warming it in a 56ºC hot water bath for 30 min to help prevent contamination in the culture (Barile 1973). Then the basal media and serum were brought to a pH of 7.65 by adding small amounts (< 1 mL) of sodium hydroxide (NaOH) solution until the media reached the desired pH. All media and serum were stored at a temperature of -30ºC and brought up to match the temperature of the glochidia before use.

2.2.5 Assessment and Identification of Contamination

Across both experiments, contamination severity was assessed daily using a scoring system. Dishes were removed from the incubator for a short time (< 10 min) and observed under the microscope to assess for contamination. The score was given before a media change or addition of Amphotericin B at approximately the same time every day. The contamination score ranged from 0–5 where a score of 0 indicated no contamination

and a score of 5 was given if contamination was so pervasive in the dish that it had to be dropped from the experiment (Table 2.2). If a dish was removed from the experiment due to severe fungal contamination (where glochidia could not be separated from the fungus), then the dish was given a score of 5 for the remainder of the experiment. At the end of both experiments, the average contamination score was calculated for each dish and used for assessment of final contamination severity.

To identify the species of fungal contamination growing in the *in-vitro* dishes (Figure 2.1), discarded samples of contaminated media from 3 *in-vitro* dishes (two 10 µg/mL dishes and one 3 µg/mL dish from Experiment 2) were poured into separate, sterile, bottles and observed under the microscope by Dr. Robert Wick (Plant Disease Diagnostic Clinic, Nematode Assay Lab, Stockbridge School of Agriculture, University of Massachusetts Amherst). All cultures had the same yeast morphology and were likely the same species; however, DNA extraction was needed to confirm identification. Using a sterile loop, samples of the fungus were spread onto petri dishes with potato dextrose agar, so there were 2 replicates of all 3 samples (1 for testing and 1 for backup). Samples were grown on the agar for 4 days in an incubator prior to DNA extraction.

DNA was extracted using the Qiagen DNeasy Plant Mini Kit and the DNA Immunoprecipitation (DIP) method. Polymerase chain reaction (PCR) was performed and the quality of the DNA was tested using standard gel electrophoresis. Samples (4 yeast, 2 filamentous and 1 negative control) were sent to the Macrogen Inc. for DNA sequencing. Upon receiving the sequences from Macrogen, the reverse complement of the sequences were taken using the tool Reverse Compliment tool (https://www.bioinformatics.org/sms/ev_comp.html). Then, the forward and reverse

nucleotides for the DNA sequences were aligned using the EMBOSS Water tool (Madeira *et al.* 2019). After the nucleotides were aligned, the DNA sequences were examined using SeaView (version 5.0.2, Gouy 2010) which identified the longest strings of nucleotides. The Basic Local Alignment Search Tool (Altschul *et al.* 1990) compared the longest nucleotide sequences to those in the database and calculated the likelihood that my sample matched samples in the database. For a more in-detail review of the methods used in fungal identification see the Standard Operating Procedure in Appendix A (Walsh 2020).

2.2.6 Experiment 1 Procedures

For Experiment 1, there were 3 treatments based on frequency of media changes: every 1 day (control, n=5), every 2 days (n=5) or every 3 days (n=3) as well as two nonexperimental dishes. Five mL of media and approximately 150 glochidia were added to each replicate petri dish (100 mm x 15 mm). During incubation, dishes were kept in a sterile incubator at 23ºC with 1.5% CO_2 and were only opened on days coinciding with media changes. All media changes took place on a sterile clean bench. During media changes, live glochidia were removed from the old media using a sterile pipette and placed in a new dish with clean media to "rinse" the glochidia. Then, the glochidia were moved to a new dish with more clean media and placed back in the incubator.

Starting on day 11 of the experiment, 10 glochidia were taken out of 2 non-experimental dishes and diluted with filtered (0.1 µm), chlorine-free water that was warmed to 23°C (to match the temperature of the incubator) to assess for transformation. One hour after full dilution, the dishes were given a fresh dose of water to promote

movement and glochidia were counted under a microscope and assessed for transformation. A glochidia was considered transformed if it was closed and dark in color, with developed adductor muscles. A glochidia was considered not transformed if it was splayed open and/or light in color without developed adductor muscles. Once at least 50% of the glochidia were considered transformed, the experimental dishes were removed from the incubator. Dishes were left on the clean bench overnight to let the glochidia acclimate and then moved to a flow through system the next day.

2.2.7 Experiment 2 Procedures

In Experiment 2, treatments varied in Amphotericin B concentration: 0 µg/mL (control), 1 µg/mL, 3 µg/mL, 5 µg/mL and 10 µg/mL. One additional treatment with 3 µg/mL was added to test a different method of Amphotericin B replenishment. Prepared glochidia were transferred to 30 dishes and divided into the 6 treatments, with 5 replicates per treatment and approximately 100 individuals in each replicate. All treatments except for the additional treatment had a media change every 3 days. The additional treatment (with 3 µg/mL of Amphotericin B) received one media change halfway through the experiment (day 6). On days 3, 9 and 12, which coincided with media changes for the other treatments, this treatment was given a 1 mL dose of a high concentration of Amphotericin B to return the dish to 3 µg/mL of active Amphotericin B.

2.2.8 Assessing Transformation

Starting on day 12 of the experiment, 10 glochidia were taken out of 2 non-experimental dishes and diluted to assess for transformation. On day 13, more than half

of the individuals moved in the diluted media after 30 min (indicating transformation), so all individuals were removed from the media. To remove from media, 1.5 mL of filtered, chlorine-free water (at pH of 7.65 and 23°C) was added to each dish. After 15 min, an additional 3 mL of water was added to each dish, then after another 15 min, 4 mL of water was added. At 45 min, using a wash bottle with the same water, the glochidia were gently spun to the center of the dish and slowly (~ 2 min) diluted from media to freshwater. The dishes were placed back in the incubator overnight and the next day 5 mL of fresh, sterile water was added to each dish. Mussels were video-recorded for 2 min using the software AmScope (version 3.7.13522). Each dish incubated for approximately 11 h before its second observation. The mussels were counted and assessed for transformation success.

2.2.9 Data analysis

2.2.9.1 Objective 1: Assessment of Contamination Severity

To compare contamination severity across different treatments, one-way ANOVAs were run using the 'aov' function in base R (R Core Team 2019). Separate relationships were analyzed for the different mitigation techniques: frequency of media changes (3 treatments), Amphotericin B concentration (5 treatments) and method of Amphotericin B replenishment (2 treatments). If there was a significant difference with multiple levels, Tukey's post hoc was used to determine if there were significant difference between groups.

2.2.9.2 Objective 2: Assessment of Transformation Success

To assess how contamination severity and various methods of mitigation influenced transformation success, I used a generalized linear mixed model (GLMM) with a logistic framework with a logit link transformation using the 'glmer' function from the 'lme4' package in R (Bates *et al.* 2015). All glochidia data were expressed as a binary response where 0 = non-transformed glochidia (i.e., failure) and 1 = transformed glochidia (i.e., success). This gave the dataset a hierarchical structure, which allowed for individual glochidia to be nested within a dish and then dish could be used as a random effect in all models (following Hazelton *et al.* 2013). To assess the effect of media change frequency on transformation success, predictors included 3 treatment levels (changes every 1, 2, or 3 days), contamination severity, an interaction term between contamination and treatment, and a random effect of dish. Daily media changes were used as the reference level for model interpretation. To assess the effect of Amphotericin B concentration on transformation success, predictors included treatment levels (0, 1, 3, 5, and 10 µg/mL), contamination severity, an interaction term between treatment and contamination, and a random effect of dish. The treatment with 0 µg/mL was used as the reference level for model interpretation. Finally, to assess the effect of Amphotericin B replenishment method on transformation success, I investigated 2 treatments that either received frequent media changes, or a dose of Amphotericin B. In model selection I also considered the parameters contamination severity, and an interaction term as well as the random effect of dish. For this analysis, the replenishment of Amphotericin B was used as the reference level for interpretation. Models were compared using Akaike Information Criteria with a correction for small sample size (AIC_c) using the 'AICc'

function in the 'MUMIn' package in R (Kamil Bartoń 2015). The parameters from all top models were assessed using the Wald statistic (*z*), standard errors, and level of significance ($p<0.05$). All model outputs were evaluated as transformation odds for each experiment (denoted as odds ratio = OR).

2.3 Results

2.3.1 Viability

The viability of dwarf wedgemussels used in Experiment 1 were 97.7%, 100.0% and 100.0%, resulting in an average viability of 99.2%. The viability of triangle floater used in Experiment 2 were 89.1%, 90.4%, 96.1%, 97.1%, 100.0% and 100.0% resulting in an average viability of 95.3%.

2.3.2 Contamination Severity

In Experiment 1, contamination severity was highest with media change every 2 days (2.61 ± 0.60) followed by every 1 day (1.36 ± 0.42) and every 3 days (0.04 ± 0.69), with significant differences between change outs every 2 versus 3 days ($p = 0.008$) (Table 2.3; Figure 2.2).

Throughout Experiment 2 there was 1 treatment that did not exhibit fungal contamination (5 µg/mL), 1 treatment that exhibited minimal contamination (1 µg/mL; only 1 replicate received a contamination score of 1 on the final day, the rest were 0), and 3 treatments with fungal contamination (0 µg/mL, 3 µg/mL and 10 µg/mL). There were 3 replicates where contamination was so severe that glochidia could not be parsed out from the contamination had to be removed from the experiment (1 dish from the 3 µg/mL

treatment and 2 dishes from 10 µg/mL treatment). Contamination severity was the lowest in dishes with an Amphotericin B concentration of 5 µg/mL (0.00 ± 0.42), followed by 1 µg/mL (0.02 ± 0.42), 0 µg/mL (0.46 ± 0.29), 10 µg/mL (1.29 ± 0.42), and 3 µg/mL (2.35 ± 0.42), with significant differences between treatments 1 µg/mL and 3 µg/mL ($p = 0.019$) and treatments 3 µg/mL and 5 µg/mL ($p = 0.018$). (Table 2.3; Figure 2.3). Contamination severity was also higher with frequent media changes (2.35 ± 0.20) than dishes that received a dose of Amphotericin B replenishment in place of a media change (0.62 ± 0.28) with significant differences between groups ($p<0.001$) (Table 2.3; Figure 2.4).

2.3.3 Fungal Identification

Standard gel electrophoresis from 4 yeast, 2 filamentous, and 1 negative control sample revealed weak bands with the Mini Kit method for the filamentous fungi, and no bands with the DIP method, so the filamentous samples were not sent out for sequencing (Figure 2.5). Sequencing results revealed a 100% identity and a 100% query match to the fungus *Candida parapsilosis* using the Qiagen DNeasy Plant Mini Kit method on the yeast sample. This fungus manifests as a yeast and in a pseudohyphae (filamentous) form (Németh *et al.* 2013).

2.3.4 Transformation Success

In Experiment 1, transformation success ranged from 3.2% to 69.4% across all treatments (n=13 dishes). Overall transformation was highest with media changes every 3 days (57.7% ± 1.8), followed by changes every 1 day (53.5% ± 6.0%), and changes every

2 days (19.0% ± 28.3%). The top model predicting transformation success included 1 random effect (dish) and 3 fixed effects: frequency of media change, contamination severity and an interaction between these 2 terms (Table 2.4). Compared to daily media changes, media changes that only took place every other day and every 3 days decreased odds of transformation by a factor of 59% and 37% per one-unit change in contamination severity, respectively (Table 2.5; Figure 2.6).

In Experiment 2, transformation ranged from 0% to 95.5%, and varied across all treatments: 0 µg/mL (91.3 % ± 4.7%), 1 µg/mL (84.0 % ± 2.1%), 3 µg/mL (69.6 % ± 39.2%), 5 µg/mL (73.5 % ± 6.1%), and 10 µg/mL (37.3 % ± 35.2%) (Figure 2.7). Model comparison indicated that the top model predicting transformation success included a random effect of dish and 3 fixed effects: Amphotericin B concentration, contamination severity, and an interaction between these 2 terms (Table 2.4). Due to a rank-deficiency, the interaction between 5 µg/mL and contamination severity was dropped from the model. Compared to the reference group (0 µg/mL), for every one unit increase in contamination severity, the odds of a glochidia transforming decreases in by 98.5%, 99.5% and 100% when they belong to treatments 1 µg/mL, 10 µg/mL and 3 µg/mL (Table 2.6; Figure 2.8).

Overall transformation was higher in dishes with a dose of Amphotericin B (93.6% ± 2.2%) compared to the dishes that received frequent media changes instead (69.6% ± 39.2%) (Figure 2.9). Model comparison indicated that the top model included 1 random effect (dish) and 3 fixed effects: method of Amphotericin B replenishment, contamination severity, and an interaction between these 2 terms (Table 2.4). Compared to the reference group (Amphotericin B replenishment dose), for every one unit increase

of contamination severity, the odds of a glochidia transforming decreases by 100% in dishes with frequent media changes (Table 2.7; Figure 2.10).

2.4 Discussion

Fungal contamination was and continues to be a problem in *in-vitro* propagation of freshwater mussels and negatively impacts transformation success (Owen *et al.* 2010). I tested methods to mitigate contamination severity in culture while maintaining high transformation success of *Alasmidonta* glochidia. Opening dishes infrequently and providing a replenishment (dose) of Amphotericin B throughout *in-vitro* culture proved to be successful in obtaining both goals. Additionally, I found that continued use of low concentrations (0–1 µg/mL) of Amphotericin B will mitigate contamination just as effectively as higher concentrations (3–10 µg/mL), which may also negatively impact transformation success. However, through a better understanding of the fungus contaminating the dishes (*Candida parapsilosis*), how it manifests in culture, impacts glochidia and its sensitivities to other antifungals, we can continue to refine our methods for contamination mitigation and propagate a higher proportion of juvenile mussels.

2.4.1 Mitigation of Contamination

Across all experiments, I was successful in my efforts to mitigate contamination in *in-vitro* culture using new and applicable methods. While investigating the impact of media change frequency, I found that dishes with media changes every 2 days experienced the most contamination. This is likely because these dishes had a high frequency of potential exposure to contamination (opening the dish), while simultaneously being exposed to contamination for longer periods of time compared to

dishes with daily media changes. Conversely, dishes that only had their media changed every 3 days were able to stay relatively sterile, because the frequency that dishes were exposed to airborne contaminants was minimized (Ryan 1994). These results indicate that media should be changed infrequently, as little as 1 time in a 13-day culture, when dishes are sterile and glochidia densities are low (43 – 57 glochidia per 3.5 mL of media) (Ma *et al.* 2018) to minimize exposure to contamination. However, it should be noted that the antifungal, Amphotericin B, depletes after 3 days in media when incubated at 37 °C (Thermofisher Scientific, personal communication). An alternative method to replenish the antifungal without a full media change is to open the dishes only to add a dose of Amphotericin B. In my study, I found that dishes that were dosed had lower levels of contamination than dishes that had frequent (every 3 days) media changes to replenish Amphotericin B, likely because there was less opportunity for exposure to contamination than with media changes.

Contamination severity also varied across different concentrations of Amphotericin B. Surprisingly, dishes with highest doses of Amphotericin B did not have the lowest contamination. The minimum inhibitory concentration needed to impede 50% (MIC_{50}) of *C. parapsilosis* rests somewhere between 0.13 µg/mL and 1 µg/mL (Tóth *et al.* 2019), meaning a concentration of 10 µg/mL should have better mitigated this fungal contamination. In my experiment, I found that higher concentrations of the antifungal, Amphotericin B does not necessarily prevent higher contamination severity, but may change how the contamination manifests. In treatments with lower Amphotericin B that experienced contamination (0 and 3 µg/mL Amphotericin B), the fungus was yeasty and covered the whole dish ubiquitously. This expression of fungal contamination was also

nearly impossible to eradicate once it appeared. In these dishes, the severity of the contamination would be lower the next day after a media change, but inevitably be just as pervasive 2 days after the media change. On the other hand, in the dishes that had a higher concentration of Amphotericin B (10 µg/mL), the fungus expressed itself in a filamentous form. This difference in fungal expression may be due to an attempt by the fungus to thrive in a more stressful environment with a higher concentration of Amphotericin B (10 µg/mL) than dishes with a lower concentration of Amphotericin B (0 and 1 µg/mL) (Tóth *et al.* 2019). The filamentous form of fungi promotes more damage to cells than yeast (Németh *et al.* 2013), as filamentous fungi can invade cells, allowing the fungi to further proliferate within the cell (Tóth *et al.* 2019).

2.4.2 Source and Mechanism of Fungal Impact

C. parapsilosis, the fungus identified in the *in-vitro* dishes, is found in hospitals (Trofa *et al.* 2008), households (Zupancic 2018), and in natural environments (Weems 1992). It is possible that the fungus appeared in the *in-vitro* dishes due to contamination exposure from human contact, as it is one of the most common fungi isolated under the fingernails of people (Trofa *et al*. 2008) and from the lab environment. However, I was wearing gloves and working under a properly sterilized clean bench every time the incubator was opened, so it also seems probable that the fungus came from the gravid mussels and transferred to their glochidia. The natural ecology of *C. parapsilosis* remains poorly understood (Kurtzman *et al.* 2011) and its natural habitat has been undefined to date (Zupancic 2018). The fungus has been documented in soil, marine (Trofa *et al.* 2008), brackish (Libkind 2017) and freshwater (Maideros 2012; Zupancic 2018) environments. Infections by the fungi have also been documented in different animal

hosts (de Cordeiro 2017). Although there have been no records of this fungus inhabiting unionids, fungi are an important component of a mussel's diet (Weber *et al.* 2017), and it is reasonable that fungus may populate in the gills and glochidia of gravid mussels.

C. parapsilosis is considered a killer yeast (Tóth *et al.* 2019), as it can produce chemicals that exert cytotoxic effects on the cells of other organisms (Efren 2014). It is known for its ability to adhere to biotic and abiotic surfaces through colonization (Tóth *et al.* 2019), which could cause further harm to its host. This fungus also proliferates in media with high levels of glucose or lipids (Pereira *et al.* 2015), which are both ingredients in the media solution (Table 2.15). After the fungus adheres itself to a surface, the fungus will form biofilm over the fungal cells which protects the cells. The biofilm then provides protection to the fungus against antifungal substances and immune responses from the host (Nett 2016; Silva *et al.* 2017). Under stressful conditions such as high CO_2, low O_2, and in the presence of a serum, a filamentous expression of *C. parapsilosis* will form (Tóth *et al.* 2019). The filamentous form has greater virulence and can more quickly and readily damage the host than the yeast form (Németh *et al.* 2013).

C. parapsilosis likely attacks the mussel glochidia in using 3 fungal enzymes: secreted aspartyl proteases, lipases, phospholipases. Not all of these enzymes are present in every strain of *C. parapsilosis*; however, they can all play a role in the destruction of host cells. Secreted aspartyl proteases (Saps) aid in the survival of fungal cells and promote damage to host cells by degrading various proteins to the host's extracellular matrix (Horváth *et al*. 2012). Lipases help *C. parapsilosis* acquire nutrients from its surroundings when exposed to a lipid-rich environment. If lipases are present, adhesion to surfaces, formation of biofilm and ultimate pathogenicity to the host increases (Tóth *et al.*

2019). Phospholipases, the least understood of all 3 enzymes, may also play a role in *C. parapsilosis'* level of virulence. It has been hypothesized that phospholipases disrupt the cell membrane of the host cell to make it easier for the fungus to attack the host (Ghannoum 2000; Kantarcioglu and Yucel 2002), although there has been no direct link between the expression of this enzyme and virulence (Tóth *et al*. 2019). As *C. parapsilosis* causes damage to its host, the fungus survives being ingested and may actively proliferate within the host cells, allowing the fungus to rapidly cause even more damage (Tóth *et al*. 2019).

2.4.3 Effects of Contamination and Mitigation Methods on Mussel Transformation

There is likely a tradeoff between opening dishes to replenish nutrients and remove contamination, and inadvertently adding new contamination when the dishes are opened. Additionally, media changes themselves may be harmful to developing glochidia by disrupting their development (Kovitvadhi *et al*. 2002; Lima *et al*. 2006; Ma *et al.* 2018). In my study, treatments that received a media change every 2 days had the highest contamination, which likely explained why it also had the lowest transformation success. In contrast, dishes that only had media changes every 3 days had very low contamination and high transformation success, this may be because the dishes were not being opened very frequently, which offered less frequent exposure to contamination, as well as less disruption to the glochidia. Daily media changes had moderate contamination and high transformation success, likely because the glochidia were getting fresh media every day, which mitigated frequent exposure to contamination. Owen (2009) tested media change frequency on the paper pondshell (*Utterbackia imbecillis*) and found dishes without media changes had lower transformation compared to mussels with media changes, a

finding that contradicts our results. However, the treatments also had different concentrations of Amphotericin By (1 vs 5 µg/L), so it is possible that the differences in transformation success could be related to the concentration of Amphotericin B rather than the frequency of media changes (Owen 2009).

The concentration of Amphotericin B influenced transformation success; dishes with high concentrations (10 µg/mL) had lower transformation success than dishes with low concentrations (0–1 µg/mL) of Amphotericin B. This may have been due to dose-limited toxicity associated with Amphotericin B (Hamill 2013). However, transformation success was generally high overall regardless of Amphotericin B concentration. This finding is consistent with Owen (2009), who found lower transformation success in dishes with higher concentrations (5 µg/mL) compared to lower (1 µg/mL) concentrations of Amphotericin B. However, as mentioned, this experiment was conducted in conjunction with media change frequencies. Ma et al. (2018) tested a much higher concentration of Amphotericin B (50 µg/mL) with the cockscomb pearl mussel (*Cristaria plicata*) and found no hinderance on transformation success when compared to much lower concentrations. These findings suggest that different mussel species may have various tolerances for antifungal and antibiotic concentrations (Ma *et al.* 2018). It is important to note that none of the prior studies evaluated contamination severity, and thus they could not assess the extent to which contamination affected transformation success across different antifungal concentrations.

The method of replenishment for Amphotericin B—full media change vs. adding additional fungicide without a media change—also influenced transformation success. Glochidia that had a replenishment of Amphotericin B were associated with higher

transformation success than those with a full media change, which may be explained by the lower contamination severity in the dishes with replenishment of Amphotericin B. In one experiment, Owen (2009) tested culture dishes that were given a 2x dose of antibiotics, including 10 µg/mL Amphotericin B, for the first 48 hours in culture, and then lowered the dose (5 µg/mL) for the duration of culture. This influx of Amphotericin B did not negatively impact transformation for the duration of the experiment; however, it also did not prevent fungal contamination (Owen 2009). The lower transformation in my experiment could also be explained by the negative effect of disturbance on developing glochidia (Kovitvadhi *et al*. 2002; Lima *et al*. 2006; Ma *et al.* 2018). Although frequent media changes reduced the risk of contamination, it can also negatively affect a cell's (or glochidia's) physiological state (Odintsova and Khomenko 1991) and possibly their ability to transform. Reducing media change frequency may promote greater transformation success in freshwater mussel propagation.

Overall, transformation success was lower in the first experiment (41.2% ± 24.8%) compared to the second experiment (74.9% ± 27.6%), potentially due to the difference in species (dwarf wedgemussel vs. triangle floater) or the timing of experiments. In the first experiment, mussels were held at the Richard Cronin Aquatic Resource Center (CARC) from March 2019 until June 2019 before they were used for propagation. Naturally, gravid dwarf wedgemussels release their glochidia as early as April (McLain and Ross 2005), so it is possible that the mussels produced less fit glochidia because they were held for too long. This decrease in fitness may have translated to lower transformation of their glochidia *in-vitro*. On the other hand, the gravid triangle floaters used for Experiment 2 were collected in the fall and held in

mussel silos in the river before propagation. Glochidia for this experiment were used for propagation in January, before they would have released naturally in the wild, which may have promoted fitness and overall transformation in this experiment. Future research is needed to determine if timing of *in-vitro* propagation and holding time of gravid mussels impacts the transformation rates of glochidia over time.

2.4.4 Recommendations for Mitigating *C. parapsilosis*

Amphotericin B has been used to control fungal contamination since *in-vitro* freshwater mussel propagation was studied by Isom and Hudson in 1982. This original recipe, which called for 5 µg/mL of Amphotericin B, has been cited and used repeatedly in experiments and *in-vitro* production of freshwater mussels (Keller and Zam 1990; Kovitvadhi *et al.* 2001; Owen 2009; Lima *et al.* 2006; Kovitvadhi 2011; Wen *et al.* 2018; Escobar-Calderón and Douda 2019). With further study, Owen (2009) found that a lower concentration of Amphotericin B, 1 µg/mL, resulted in a higher proportion of transformed juveniles, which led to use of lower concentrations (0.67 µg/mL – 1 µg/mL) of Amphotericin B (Monte McGregor, personal communication). Broad spectrum antifungals often fail to eliminate target organisms (Leifert and Cassells 2001) and additional research should be done to determine if the same species of fungi are infecting *in-vitro* propagation dishes in labs across the country to better tailor antifungal use (Ryan 1994). Identification of fungi can lead to better mitigation strategies and tailored antifungals to target specific fungi.

The prevention of fungal contamination may require experimentation with other drugs not yet tested with *in-vitro* freshwater mussel propagation. To prevent an infection by *C. parapsilosis*, three echinocandin drugs—caspofungun, micafungin and

andulafungin— have been recommended as the first line of defense (Tóth *et al.* 2019). Echinocandins are synthetically modified lipopeptides from fermented broths of a variety of fungi (Cappelletty and Eiselstein-McKitrick 2007) and are the most effective class of antifungals for *C. parapsilosis* (Pappas *et al.* 2016). Echinocadins were not tested by Owen *et al.* (2010) and there is no information about their use with freshwater mussel *in-vitro* propagation. Testing of these drugs with mussels should consider the minimum concentration to inhibit the growth of 50% of the fungus (MIC_{50}) for each drug (Caspofungin MIC_{50} = 0.85–2 µg/mL, micafungin MIC_{50} = 1 µg/mL, and adidulafungin MIC_{50} = 2 µg/mL; Tóth *et al.* 2019) to prevent development of immunity (Tóth *et al.* 2019). Although all of these drugs can help inhibit *C. parapsilosis*, once biofilm forms on biotic and abiotic surfaces, the biofilms can lower the effectiveness of the antifungals (Tóth *et al.* 2019).

Fungal contamination can and should be mitigated beyond the use of antifungals and antibiotics. Contamination should be moderated by using the building blocks for contamination mitigation (Ryan 1994). These building blocks include good aseptic techniques, good housekeeping, use of healthy cells (glochidia), strategic use of antibiotics, understanding the origins of the contamination, and consistent monitoring of the contamination (Ryan 1994). Good aseptic techniques and good housekeeping include the maintenance of a clean workspace where *in-vitro* culture takes place. Methods may include the use of a laminar flow hood, a UV light and 75% ethanol for sterilization of the clean bench and equipment. Additionally, because freshwater bivalves are filter feeders, they harbor microorganisms like fungus and bacteria, so it is important to wash the mussel inside and out (Quinn *et al.* 2009) before collecting glochidia. Some lab

practices call for washing the shell of the gravid mussels with diluted bleach, and then placing the mussel in 1–2 changes of sterile filtered (0.45-µg) water for one hour before extracting glochidia (Kern 2017). Other, more drastic, measures include sacrifice of the gravid mussel and removal of the gills to help promote sterility (Kern 2017); however, this practice may not be appropriate for rare species. Cleaning the gravid mussel inside and out can help minimize presence of microbial contamination (Ryan 1994) on the glochidia at the onset for a cleaner culture. Another building block for preventing contamination includes strategic use of only high quality glochidia, which is a practice used in many *in-vitro* propagation facilities (Monte McGregor, personal communication). If a gravid mussel has low viability, then it may not be used for culture because it may have low quality glochidia and could cause contamination issues in the dishes. Strategic use of antibiotics and knowing the origin of the contamination are also important considerations. Broad spectrum antifungal compounds often fail to eliminate target organisms (Leifert and Cassells 2001). Knowing where the contamination came from and what species it is can lead to better diagnosis and treatment of the fungal contamination. Finally, by monitoring dishes for contamination daily and closely, the effect of contamination on transformation success can be minimized through media changes.

Other recommendations for reducing contamination include only opening the dish if you see contamination in the dish, and otherwise leaving it closed (i.e., not opening the dish to pick out dead glochidia). The use of Amphotericin B in low doses, or perhaps not at all, may also mitigate fungal contamination just as effectively as higher concentrations. High concentrations of Amphotericin B were not directly related to lower contamination severity in my study. If Amphotericin B is going to be used, adding doses of

Amphotericin B to the dish instead of executing frequent full media changes may help lessen chance of contamination and increase transformation success (Kovitvadhi *et al.* 2002; Lima *et al.* 2006). This technique may have to be used in conjunction with low densities (43 - 57 glochidia per 3.5 mL of media) so nutrient requirements for the glochidia are met (Ma *et al.* 2018). Different propagation facilities may require different techniques due to variation in species of contamination, equipment, sterility of the environment and number of available staff. Labs likely need to develop lab-specific protocols to transform the highest proportion of juvenile mussels for their facility.

2.5 Conclusion

This study offers new ideas for improving *in-vitro* mussel propagation techniques that potentially allow for the better control of fungal contamination and thus higher transformation success. Using higher (3–10 µg/mL) concentrations of Amphotericin B than currently recommended (Owen *et al.* 2010) does not necessarily prevent contamination, and instead infrequent media changes (unless significant contamination appears) may promote greater transformation success. Furthermore, I found that adding doses of Amphotericin B in place of frequent media changes may reduce exposure to contamination and minimize disturbance to developing glochidia (Odintsova and Khomenko 1991; Kovitvadhi *et al*. 2002; Lima *et al*. 2006; Ma *et al.* 2018), which may promote higher transformation success of juveniles. By using glochidia more efficiently with *in vitro* propagation compared to *in-vivo* propagation (i.e., maximizing the number transformed into juveniles), we have the potential to propagate a higher number of juvenile mussels, which is especially important for freshwater mussels with a low

fecundity (Lima *et al.* 2012). Further research is needed to determine what species and types of contamination are infecting mussel propagation facilities. With identification of fungus, the mitigation of contamination can be targeted more specifically using different antifungals. Through the establishment of new and different methods to better control fungal contamination, we could promote a lower barrier to entry for cost and equipment for the propagation for freshwater mussels. Although this research only considered *in-vitro* propagation of two *Alasmidonta* species, the information gained will likely offer value for propagation efforts for other freshwater mussel species.

Table 2.1. Ingredient list for media solution, based on Owen *et al.* (2010).

Ingredient	Quantity
M199 powder	10 g
D-(+) – Galactose	2.6 g
D- (+) – Glucose	2.0 g
99% L-Ornithine monohydrochloride	2.5 g
L-Taurine	40 mg
MEM Nonessential Amino Acid solution	0.75 mL
MEM Amino Acid solution	1.5 mL
Carbenicillin disodium salt	200 mg
Gentamicin sulfate salt	200 mg
Rifampicin	200 mg
Amphotericin B powder	1 mg
Lipid Mixture	1.5 mL
MEM Vitamins	1.5 mL
Menhaden oil	1.5 mL
Chlorine free water	1500 mL

Table 2.2. Contamination severity scores and their descriptions.

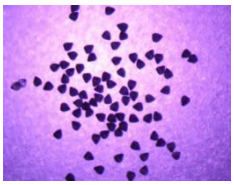
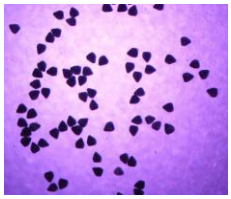
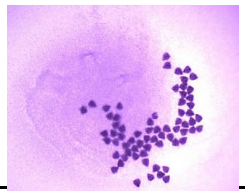
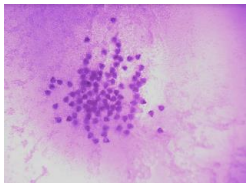
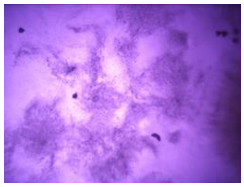
Contamination Score	Description	Example Picture
0	No fungus detectable in the dish	
1	Fungus can only be observed under a compound light microscope	
2	Fungus visible with the naked eye only when you swirl the dish around. It creates a darker spot around the glochidia when the dish is swirled, like a small eye of a storm.	
3	Fungus evident without swirling the dish around.	
4	Fungus is pervasive throughout the dish.	
5	Fungus is intertwined with the glochidia, that the two cannot be separated and the dish had to be removed from the experiment	

Table 2.3. One-way analysis of variance results explaining contamination severity across all methods of contamination mitigation. Contamination severity is measured as average contamination score

Method of mitigation	n	Sum of Squares	df	Mean Square	F	p
Frequency of media change	13	12.65	2	6.32	-16.01	0.012
Amphotericin B concentration	25	20.12	4	5.03	-21.74	<0.001
Method of Amphotericin B replenishment	10	7.55	1	7.55	38.26	<0.001

Table 2.4. Comparison of generalized linear mixed models for predicting transformation success with a binomial distribution and a logit link transformation. All models included a random effect of dish. Contamination severity was measured as average contamination score across all days. *K* = number of parameters, ΔAIC_c = change in Akaike Information Criterion with a correction for small sample sizes, and LL = log likelihood.

All Models	*K*	ΔAIC_c	Weights	LL
Frequency of media change models (Experiment 1)				
Frequency of media change * Contamination + Frequency of media change + Contamination	7	0.00	0.78	-10.64.5
Frequency of media change + Contamination	5	2.54	0.22	-1067.8
Contamination	3	20.50	<0.01	-1078.8
Frequency of media change	4	35.50	<0.01	-1085.3
Null	2	42.68	<0.01	-1090.9
Amphotericin B concentration models (Experiment 2)				
Amphotericin B Conc. * Contamination + Amphotericin B Conc. + Contamination	10	0.00	1.00	-936.95
Amphotericin B Conc. + Contamination	7	30.16	<0.01	-955.06
Contamination Severity	3	60.86	<0.01	-974.42
Amphotericin B concentration	6	65.74	<0.01	-973.85
Null	2	70.06	<0.01	-980.02
Method of Amphotericin B replenishment models (Experiment 2)				
Method * Contamination + Method + Contamination	5	0.00	1.00	-234.75
Method + Contamination	4	24.29	<0.01	-247.90
Contamination	3	27.44	<0.01	-250.48
Method	3	32.31	<0.01	-252.92
Null	2	32.90	<0.01	-254.22

Table 2.5. Top generalized linear mixed model for assessing how glochidia transformation is affected by frequency of media changeouts, contamination severity, and an interaction. CI = confidence interval, LO = Log Odds, OR = Odds Ratio. N= 13 dishes.

Variable	Log Odds	Odds Ratio	CI 2.5% (LO)	CI 97.5% (LO)	CI 2.5% (OR)	CI 97.5% (OR)	Odds Ratio (Percent Change) %	z-value	p-value
Intercept (every 1 day)	0.39	1.48	-0.48	1.27	0.62	3.56		0.94	0.348
Every 2 days	0.44	1.55	-0.51	1.39	0.60	4.01		0.96	0.336
Every 3 days	-0.04	0.96	-0.96	0.86	0.38	2.36		-0.10	0.921
Intercept (Contamination)	-0.18	0.83	-0.82	0.44	0.44	1.56		-0.61	0.539
Every 2 days * Contamination	-0.90	0.41	-1.55	-0.25	0.21	0.78	-59.0 %	-2.89	0.004
Every 3 days * Contamination	-0.47	0.63	-3.67	2.82	0.03	16.75	-37.0 %	-0.30	0.762

Table 2.6. Top generalized linear mixed model for assessing how glochidia transformation is affected by Amphotericin B concentration in media (LO = Log Odds, OR = Odds Ratio). N = 25 dishes.

Variable	Log Odds	Odds Ratio	CI 2.5% (LO)	CI 97.5% (LO)	CI 2.5% (OR)	CI 97.5% (OR)	Odds Ratio (Percent Change) %	z-value	p-value
Intercept (0 µg/mL)	2.15	8.57	1.39	2.91	4.02	18.31		5.55	<0.001
1 µg/mL	-0.42	0.66	-1.27	0.43	0.28	1.54		-0.96	0.336
3 µg/mL	15.69	6.52E+6	8.32	23.06	4121.71	1.32E+10		4.18	<0.001
5 µg/mL	-1.11	0.33	-1.93	-0.29	0.14	0.75		-2.65	0.008
10 µg/mL	-0.91	0.40	-1.83	0.01	0.16	1.01		-1.94	0.053
Intercept (Contamination)	0.6	1.82	-0.78	1.98	0.46	7.25		0.85	0.394
1 µg/mL * Contamination	-4.14	0.015	-15.34	7.06	0.00	1162.37	-98.5 %	-0.73	0.469
3 µg/mL * Contamination	-8.04	0.00	-11.71	-4.37	0.00	0.01	-100.0 %	-4.30	<0.001
10 µg/mL * Contamination	-2.93	0.05	-4.56	-1.30	0.01	0.27	-99.5 %	-3.52	<0.001

Table 2.7. Top generalized linear mixed model for assessing how glochidia transformation is affected by method of Amphotericin B replenishment in media (LO = Log Odds, OR = Odds Ratio). N = 10 dishes.

Variable	Log Odds	Odds Ratio	CI 2.5% (LO)	CI 97.5% (LO)	CI 2.5% (OR)	CI 97.5% (OR)	Odds Ratio (% Change)	z-value	p-value
Intercept (Replenishment)	2.84	17.03	2.01	3.76	7.45	42.96		6.38	<0.001
Frequent media change	13.96	1.15E+6	9.63	21.75	1.52E+4	2.79E+9		4.80	<0.001
Intercept (Contamination)	-0.27	0.76	-1.38	0.89	0.25	2.43		-0.48	0.635
Frequent media change * Contamination	-6.68	0.00	-10.25	-4.39	0.00	0.01	-100.0	-4.62	<0.001

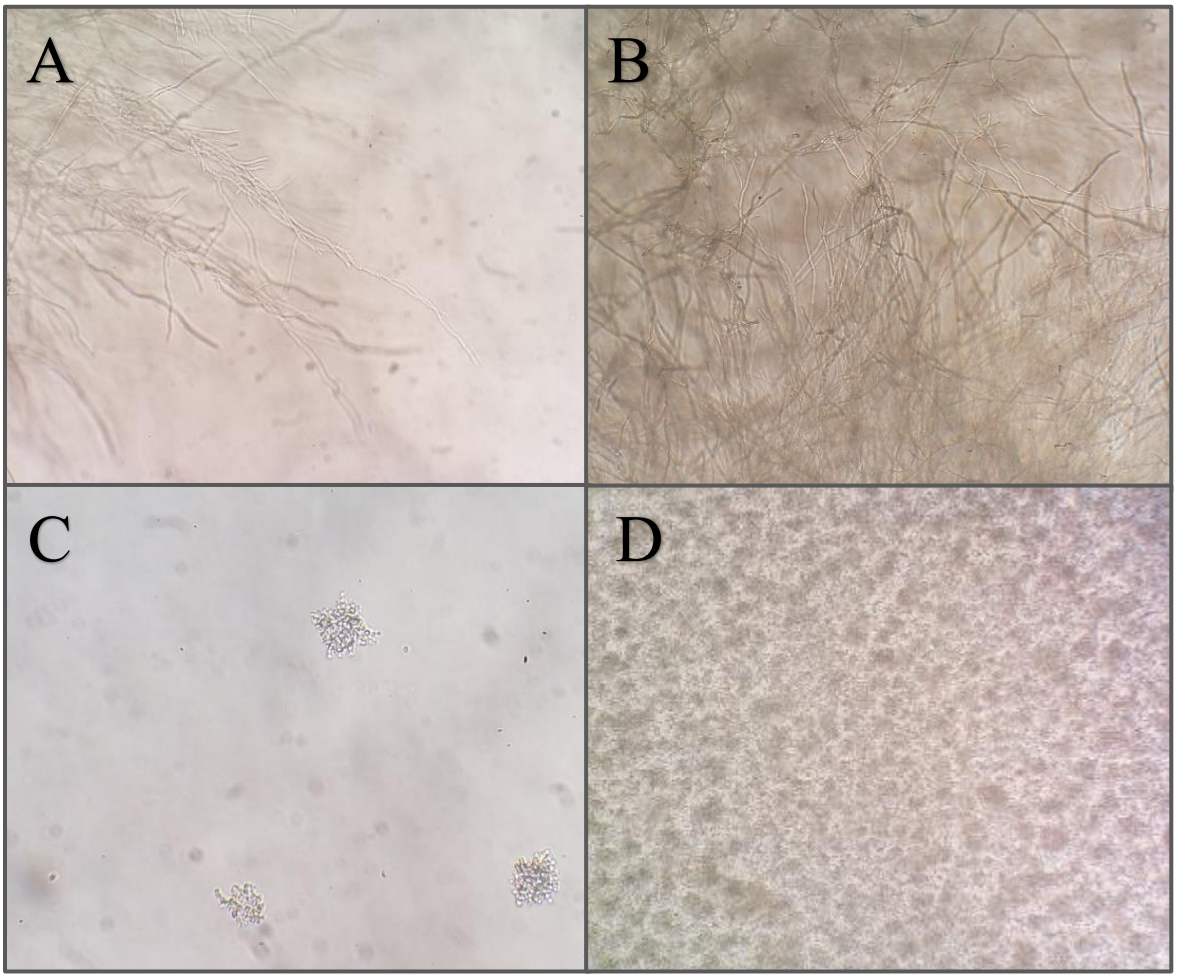


Figure 2.1. Filamentous (pseudohyphal) and (panels A and B) and yeast expressions (panels C and D) of *C. parapsilosis*. All images are *200x* magnification.

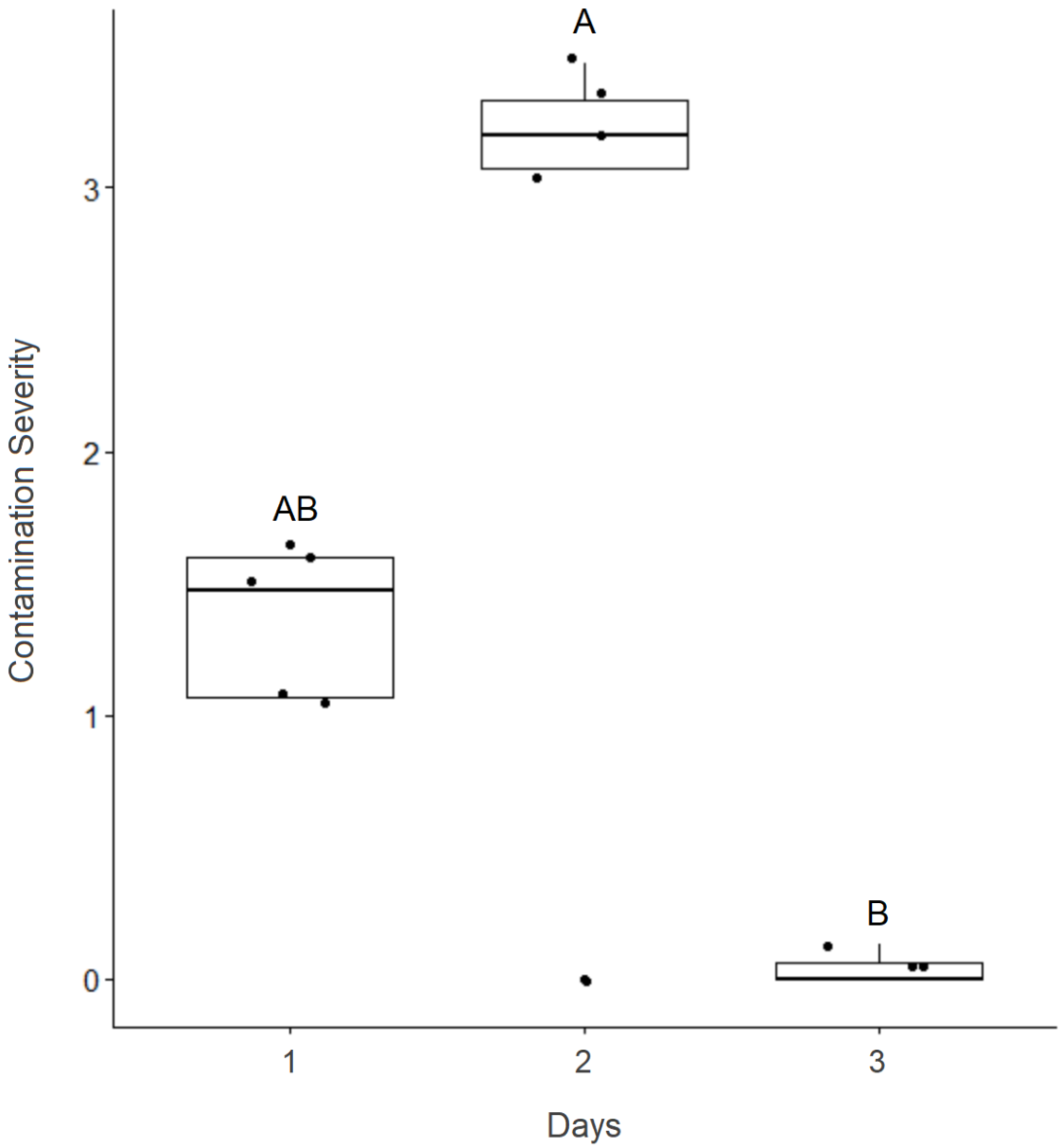


Figure 2.2. Boxplot (median and interquartile; whiskers = min and max value) of contamination severity across media change frequencies from Experiment 1 (every 1 day = 5 replicates, every 2 days = 5 replicates, every 3 days = 3 replicates). Significant differences between groups represented by different letters.

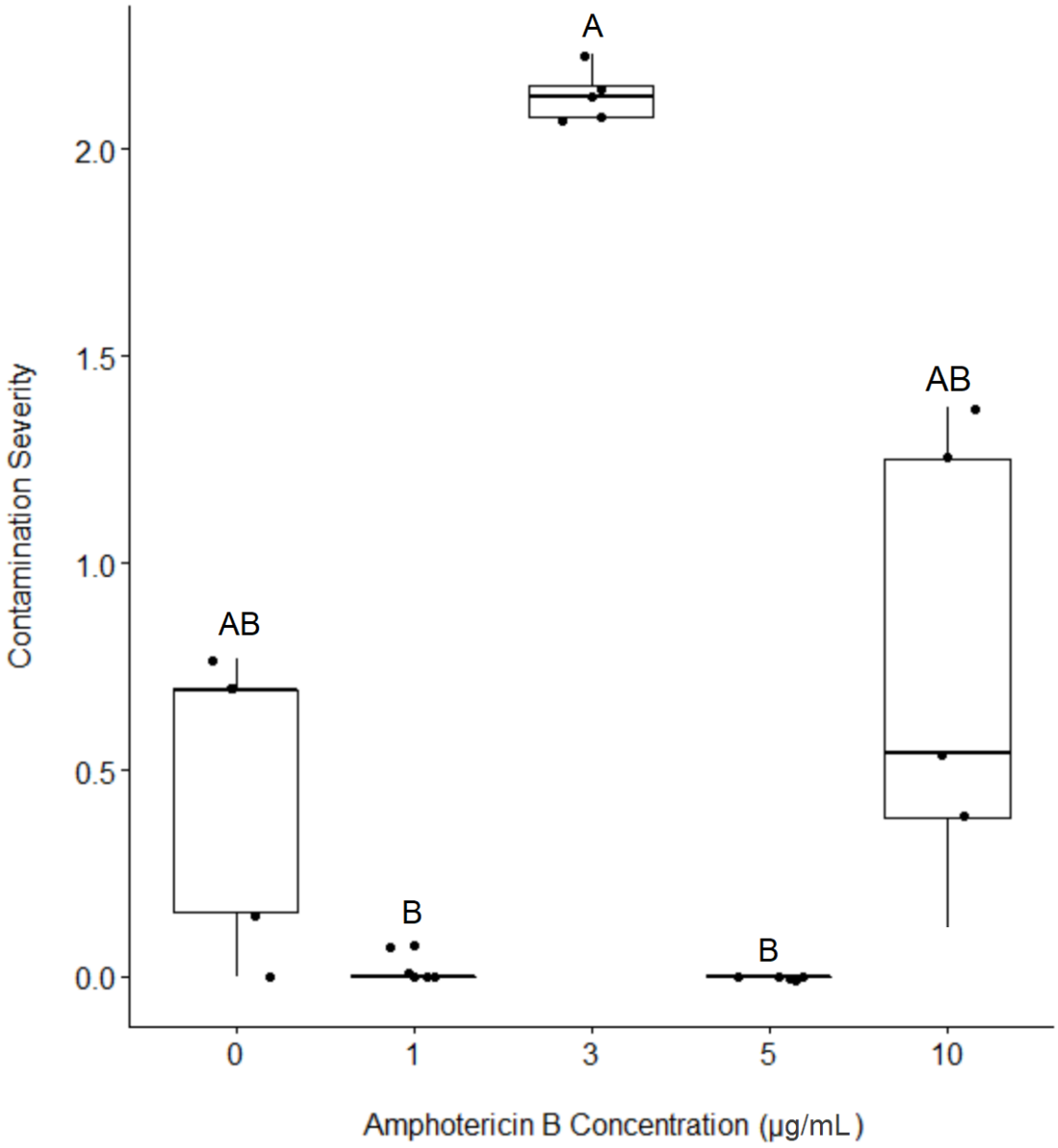


Figure 2.3. Boxplot (median and interquartile; whiskers = min and max value) of contamination severity across concentrations of Amphotericin B (n= 5 treatments per replicate). A one-way analysis of variance revealed an effect of treatment ($F= 42.58$, $p< 0.001$), and significant differences between treatments based on a Tukey's post hoc test are represented by different letters.

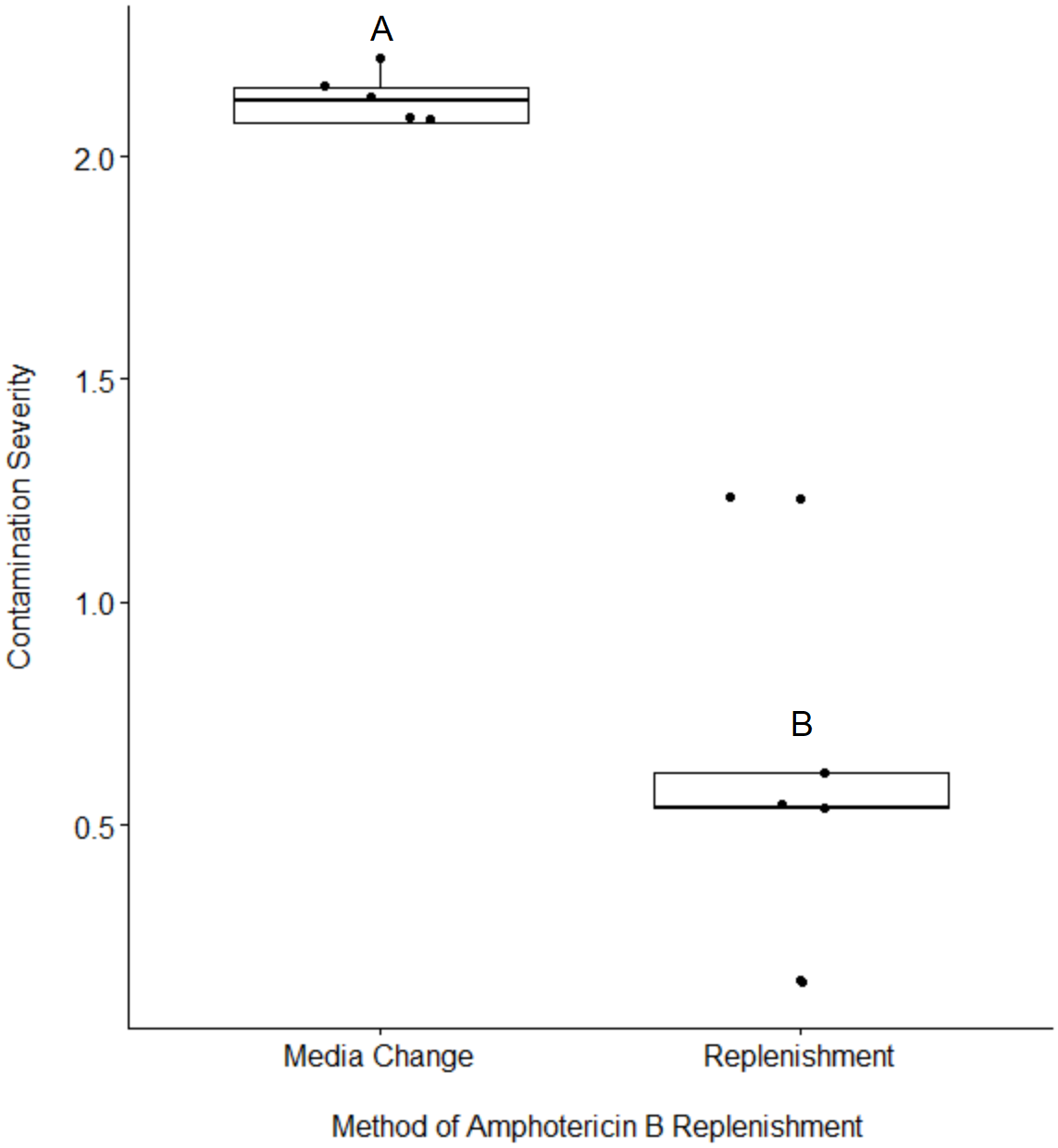


Figure 2.4. Boxplot (median and interquartile; whiskers = min and max value) of contamination severity across methods of Amphotericin B replenishment. Both treatments had an Amphotericin B concentration of 3 µg/mL. The "Media Change" treatment received a media change every other day while the "Replenishment" treatment only received one media change on day 6 of the experiment, and otherwise received a dose of Amphotericin B. A one-way analysis of variance showed differences between these groups ($F = 74.28$, $p < 0.001$).

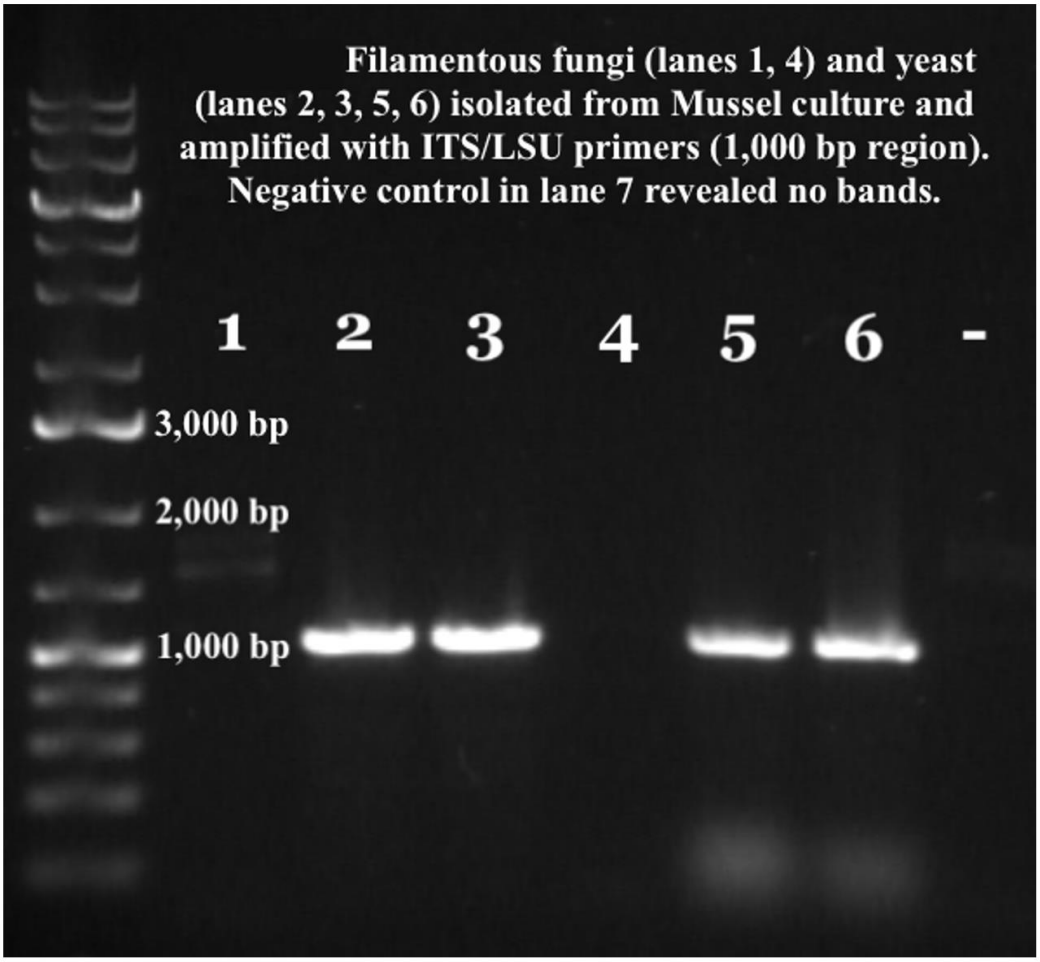


Figure 2.5. Gel electrophoresis results for the amplification for the DNA of the fungus, *C. parapsilosis*.

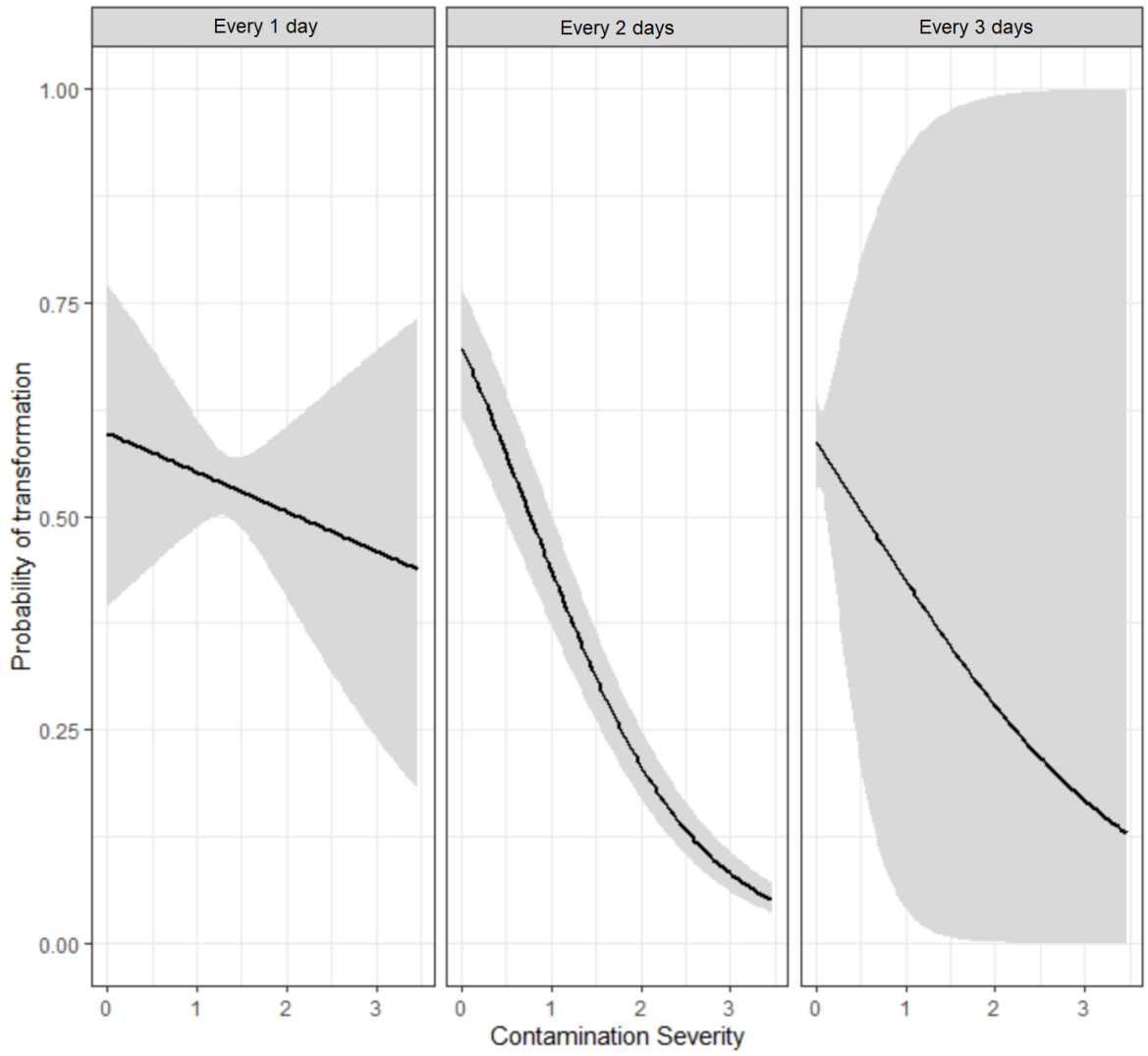


Figure 2.6. Probability of transformation across different frequencies of media changes across different levels of contamination severity (n = every 1 day, 5 replicates, every 2 days = 5 replicates, every 3 days = 3 replicates).

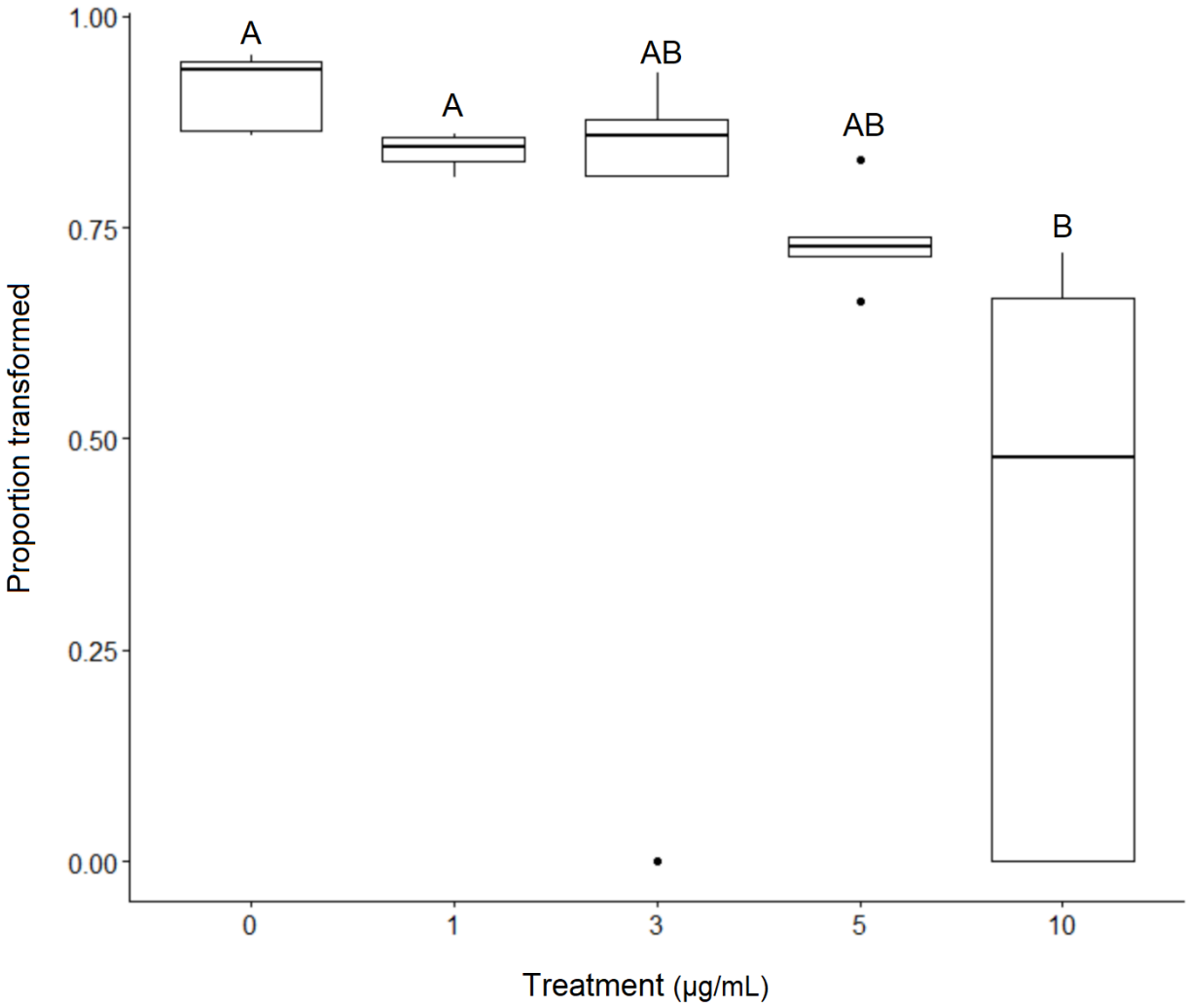


Figure 2.7. Boxplot (median and interquartile; whiskers = min and max value) of proportion of transformation of glochidia across different concentrations of Amphotericin B (n =5 replicates per treatment). A one-way analysis of variance revealed differences among treatments (F= 3.798, p = 0.019) and significant differences between individual treatments based on a Tukey's post hoc test are represented by different letters.

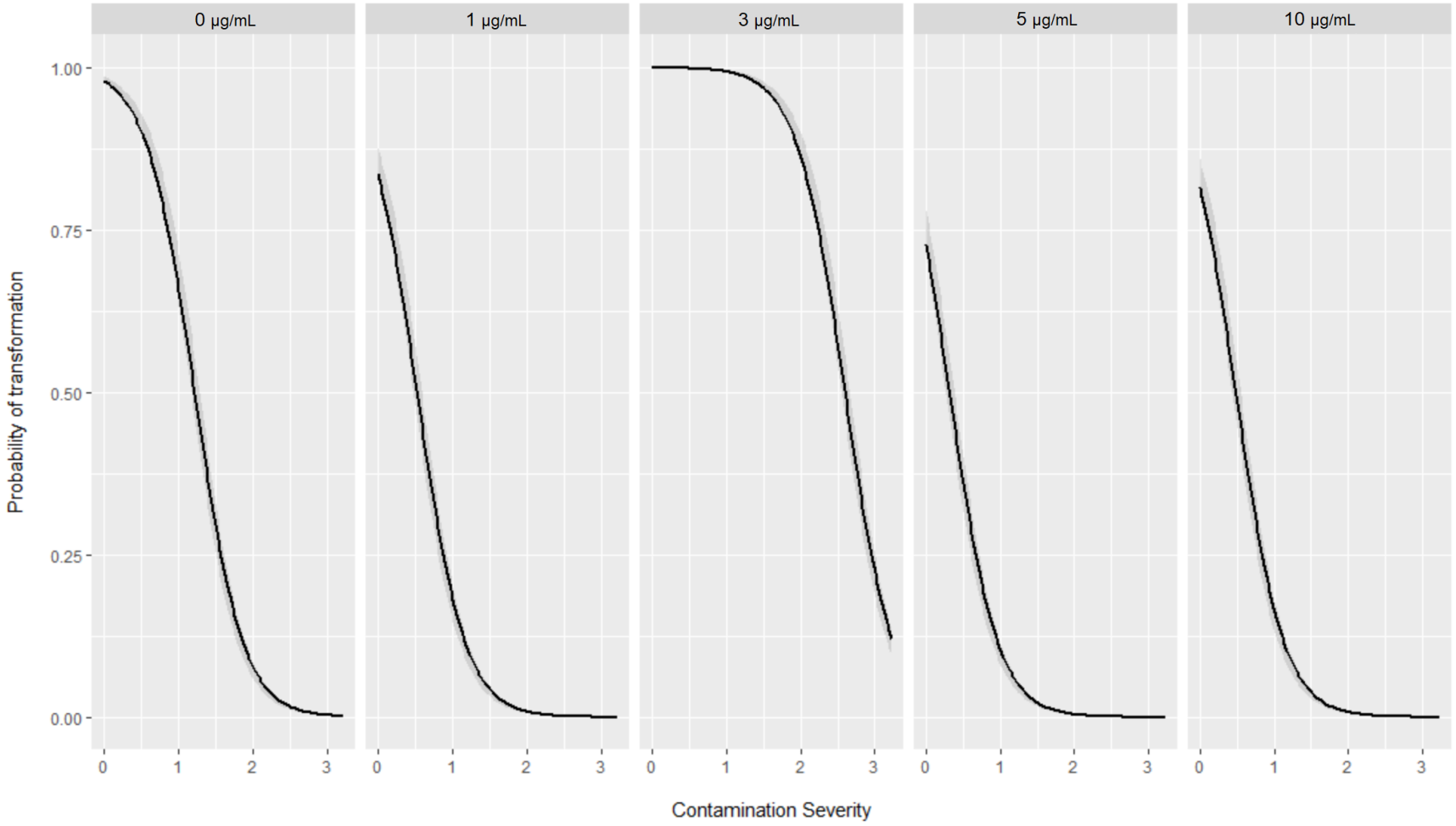


Figure 2.8. Bootstrapped data (1,000) indicating how probability of transformation is affected by contamination severity across different treatments of Amphotericin B (n = 5 replicates per treatment).

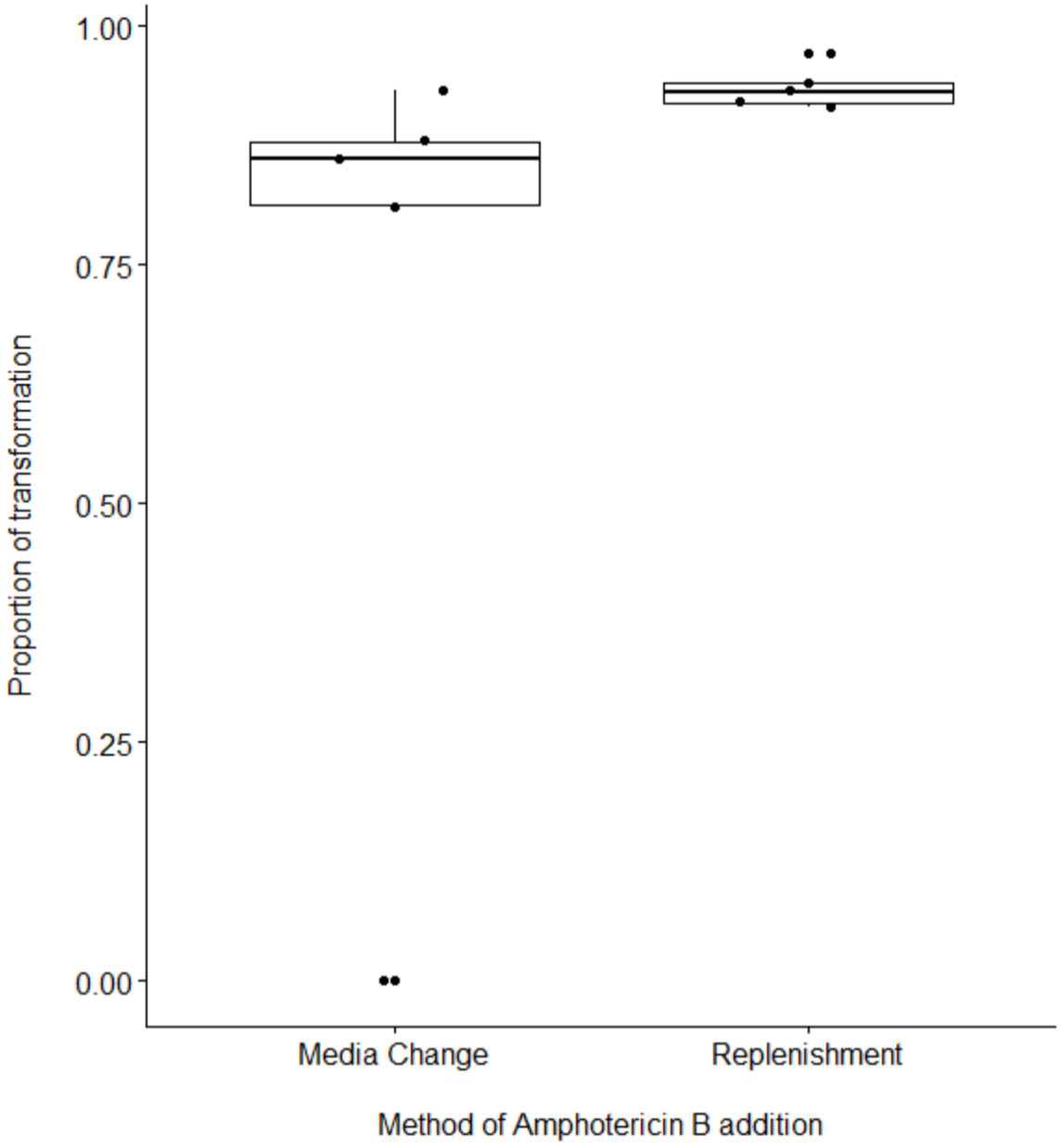


Figure 2.9. Boxplot (median and interquartile; whiskers = min and max value) of proportion of transformation of glochidia across different methods of replenishing Amphotericin B (n= 5 replicates per treatment). A one-way analysis of variance determined there was no significant difference between these groups (F =1.86, p = 0.21).

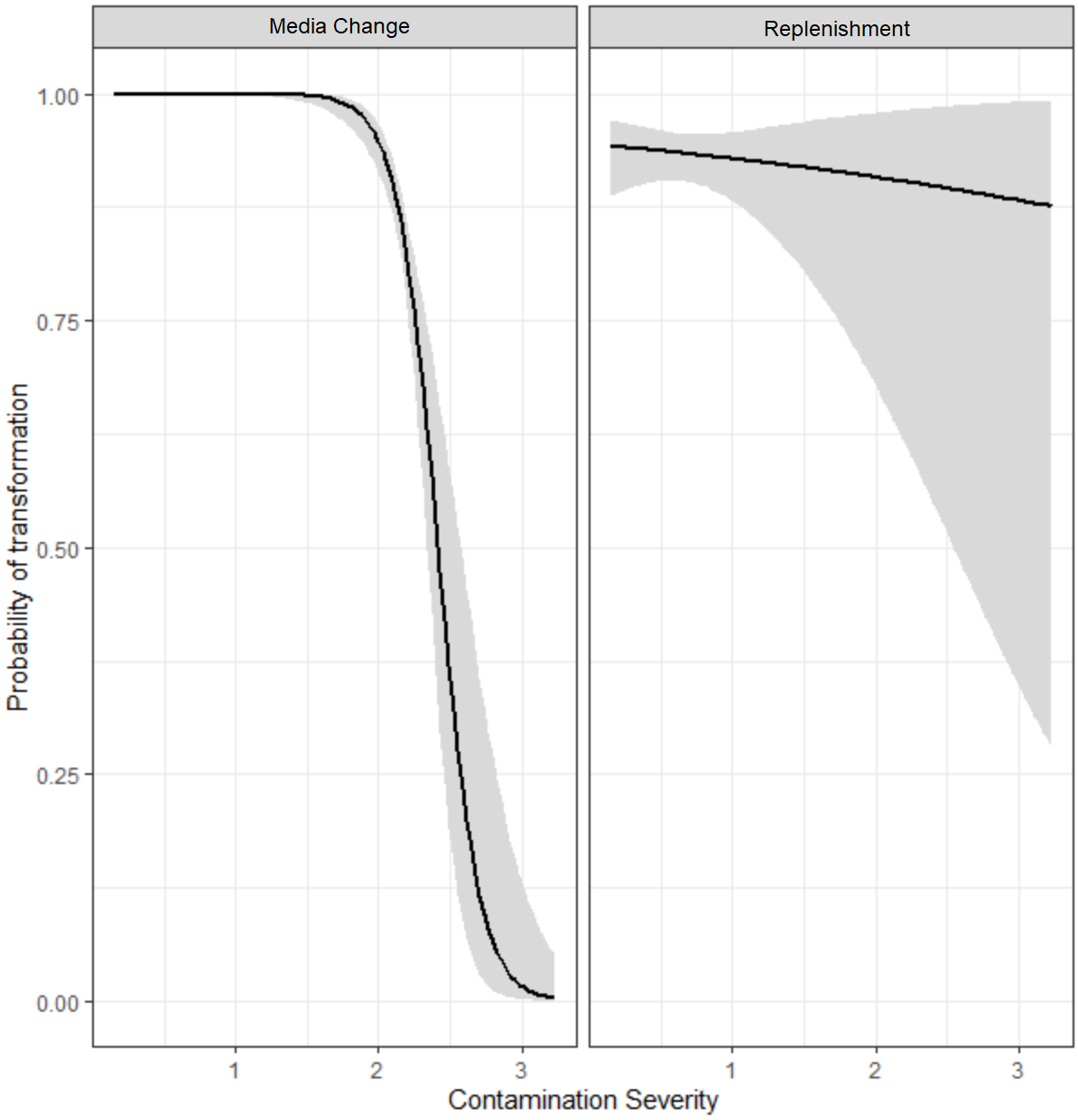


Figure 2.10. Probability of transformation for different methods of Amphotericin B application across different levels of contamination severity (n = 5 replicates per dish).

CHAPTER 3

ASSESSING HOST FISH ASSEMBLAGES NEAR DWARF WEDGEMUSSEL POPULATIONS

3.1 Introduction

Freshwater mussels require a host fish in order to reproduce and disperse (Haag 2012), which are both critical for maintaining healthy populations. Due to their sedentary nature, freshwater mussels often live in discrete patches within a river system (Strayer 2008), and thus dispersal of glochidia (larvae) between discrete populations is critical for maintaining genetic variability (Irmscher *et al.* 2015). Dispersal of glochidia upstream (using host fish) and downstream (using host fish and current) allows mussels to move into previously unoccupied areas, which expands their range and allows new populations establish (Strayer 2008). Dispersal also connects populations within a geographic area and allows the maintenance of metapopulations through genetic mixing (Strayer 2008). Freshwater mussels may use a combination of migratory and local host fish to promote genetic diversity and persistence (Fritts *et al.* 2012). A lack of migratory hosts can compromise the genetic integrity of mussels (Schwalb *et al.* 2010) and indicate that a mussel population is isolated (Vaughn 2012), which could lead to genetic constraints (Reagan 2008).

For the dwarf wedgemussel (*Alasmidonta heterodon*), several host fish have been identified and successfully transform dwarf wedgemussel glochidia in the lab (St. John White *et al.* 2017), however, further clarification is needed on which of these fishes operate as natural hosts in the wild. Dwarf wedgemussel have long been considered to be host-specialists (Haag 2012); however, laboratory studies conducted by St. John White *et*

al. (2017) found that 8 species from 5 families exhibited varying degrees of transformation success of dwarf wedgemussel glochidia (Table 3.1), suggesting that the dwarf wedgemussel is a host generalist. Specifically, dwarf wedgemussel transformed on 3 migratory host fish: Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), and striped bass (*Morone saxatilis*) and 5 local host fish: tessellated darter (*Etheostoma olmstedi*), slimy sculpin (*Cottus cognatus*), shield darter (*Percina peltata*), mottled sculpin (*Cottus bairdii*) and banded killifish (*Fundulus diaphanus*) (St. John White *et al.* 2017). While this shows that glochidia transformation is possible on several fish species, host use in the wild may not be what a laboratory study would predict (Haag 2012).

Information on dwarf wedgemussels and their potential host fish in the wild is fairly limited. Dwarf wedgemussel glochidia have only been observed on tessellated darter in the wild (McLain and Ross 2005); however, the importance of host fish in the persistence of mussel populations has been documented in other mussel species (Vaughn and Taylor 2000). While several studies have linked fish species richness and abundance to mussel diversity (Haag and Warren 1998, Vaughn and Taylor 2000, Schwalb *et al.* 2013, Negishi *et al*. 2014, Daniel *et al.* 2018), few studies have specifically investigated relationships between fish abundance and generalist mussel species (Douda *et al.* 2012, Daniel *et al*. 2018). One study on generalist species found a positive association between host fish richness and distribution of mussel species, including the elktoe (*Alasmidonta raveneliana*) and slippershell (*Alasmidonta viridis*) (Daniel *et al*. 2018), which are both closely related to the dwarf wedgemussel. Dwarf wedgemussels and their relationship to other potential host fish (as studied by St. John White *et al.* 2017) in the wild is still largely unknown.

Understanding host fish–mussel relationships is also critical for mussel restoration (Strayer 2019). Mussel propagation projects have greatly improved over the last few years, which have allowed the production of juvenile mussels by the thousands (Patterson *et al.* 2018, Strayer *et al.* 2019). Before releasing propagated juvenile mussels into potential restoration locations, it is important to confirm availability of adequate numbers of host fish for reproduction, along with adequate water quality and habitat (Strayer *et al.* 2019). However, without understanding which fishes are positively associated with mussel species and which fishes may act as hosts in the wild, researchers cannot confidently release propagated individuals in the wild.

This study aimed to understand links between fish abundance and dwarf wedgemussel abundance and occurrence. Specifically, my study objectives were to 1) characterize fish assemblages near dwarf wedgemussel locations and unoccupied locations, 2) determine if dwarf wedgemussel occurrence and abundance are related to abundance of the known host fish species, tessellated darter (McLain and Ross 2005) and other potential host fish species (per St. John White *et al.* 2017). This research will be used in finding potential augmentation sites for dwarf wedgemussel to help the recovery of the species.

3.2 Methods

3.2.1 Study Area

The dwarf wedgemussel ranges from the Neuse River in North Carolina to the Connecticut River on the border of New Hampshire and Vermont (USFWS 2019). This study focused on sites with known dwarf wedgemussel populations in the Connecticut

River watershed and in the northern part of the Delaware River watershed (Figure 3.1). The 670-km Connecticut River begins in Quebec, Canada and flows to the Long Island Sound (Garvine 1975). The mainstem of the Upper Connecticut River has one of the largest remaining populations of dwarf wedgemussels throughout its range; however, dwarf wedgemussel are predominantly found in the tributaries of the Connecticut River watershed (Nedeau 2008). In the Delaware watershed, all remaining dwarf wedgemussel populations persist in the mainstem and tributaries of the upper portion of the watershed in New York, New Jersey, and Pennsylvania. Unlike the mainstem of the Connecticut River, which has 16 dams along its main channel (Clay 2006), the entire 531-km of the mainstem of the Delaware River is free flowing (Brown *et al.* 2005).

3.2.2 Mussel and Fish Data Compilation

Dwarf wedgemussel point location data from 1997–2019 were compiled from the U.S. Geological Survey (Heather Galbraith, Leetown Science Center and Barry Baldigo, New York Water Science Center), U.S. Fish and Wildlife Service (Melissa Grader, New England Field Office), and Massachusetts Division of Fisheries and Wildlife. Fish survey data from 1999–2019 were collected from New Hampshire Fish and Game, Connecticut Department of Energy and Environmental Protection, Massachusetts Division of Fisheries and Wildlife, New Jersey Department of Environmental Protection and the U.S. Geological Survey (Barry Baldigo, New York Water Science Center).

Mussel and fish location data were mapped in ArcMap (version: 10.7.1, ESRI 2019, Redlands, CA). Only fish collections within 500 m upstream or downstream of the mussel location, on the same body of water (e.g., not a tributary stream), and without a

barrier (e.g., dam) between fish and mussel locations were included. Additionally, only fish surveys that were collected using electrofishing equipment (boat, barge or backpack), included specified effort (in shock seconds), and where samples targeted the entire fish assemblage were included.

3.2.3 Fish Collection

Where no fish assemblage information was available that met the criteria, I worked with state agencies to sample fishes near dwarf wedgemussel locations. All fish surveys (1 in New Hampshire, 3 in Connecticut, 5 in New Jersey and 7 in Massachusetts) took place in summer (June to August) 2019 during baseflow conditions with low turbidity to provide safe electrofishing conditions, easy maneuverability, and high detection of fishes. Reaches were selected based on accessibility and wadeability (i.e., < 1 m deep) within 500 m of known dwarf wedgemussel locations. Fishes were sampled using 1 or more (if > 8 m wide) backpack or barge electrofishing equipment in a single, upstream pass without block nets. Fish were captured in small dip nets and temporarily placed in aerated 5-gal buckets until they were identified, counted, measured for length and weight, and released. For all fish surveys catch-per–unit-effort (CPUE) for each fish species' was calculated using shock seconds.

3.2.4 Data Analysis

3.2.4.1 Objective 1: Fish assemblages

I used one-way analysis of variance (ANOVA) to test for differences in fish richness, fish abundance, host fish richness and host fish abundance between dwarf wedgemussel presence and absence locations. Fishes that were considered host fish were brown trout, tessellated darter, slimy sculpin and shield darter. Analyses were conducted separately in each watershed.

Differences in overall fish assemblages among survey sites were assessed using nonmetric multidimensional scaling (NMDS) using the Bray Curtis similarity index (Bray and Curtis 1957). All fish, including rare species, were included in the analyses. Analyses were performed using the 'vegan' package (Oksanen *et al.* 2011) in R. I assessed overall dissimilarity between watersheds (Connecticut vs Delaware) and dwarf wedgemussel occurrence (presence vs absence) in both watersheds and in only the Delaware River watershed using the 'adonis' function. A similarity percentages analysis (Clark 1993) was conducted using the 'simper' function (Oksanen *et al.* 2011) to determine which fish species were driving the dissimilarities between dwarf wedgemussel presence and absence locations in both watersheds combined and in the Delaware River watershed. For the top-contributing species (>3% contribution), I calculated percent contribution and *p*-values which were adjusted for multiple comparisons using False Discovery Rate method (Benjamini and Hochberg 1995; Nessimian *et al.* 2008).

3.2.4.2 Objective 2: Predicting Dwarf Wedgemussel Occurrence and Abundance

To determine how dwarf wedgemussel occurrence related to host fish abundance, I ran a generalized linear mixed model (GLMM) with a binomial distribution and a complementary log-log link transformation (Baddeley *et al.* 2010, Elliot *et al.* 2018) using the 'glmer' function in the 'lme4' package in R (Bates *et al.* 2015). I tested collinearity among host fish abundance (brown trout, tessellated darter, slimy sculpin and shield darter) using scatterplot matrices. Since no strong correlation among fish abundance variables was found (e.g. Pearson's $r < 0.4$), predictors included all combinations of host fish species. I ran analyses for both watersheds combined (with a random effect of watershed) and Delaware River watershed only (with a random effect of stream). For the analysis considering both watersheds, I only included tessellated darter and brown trout abundances, as they were the only host fish that appeared in both watersheds. Models were ranked using Akaike Information Criteria with a correction for small sample size (AICc) using the AICtab function in the AICmodavg package in R (Mazerolle 2017). Model fit was compared based on delta AICc model weights and log likelihood values. Model validation for the top model was analyzed against a reduced model using the likelihood ratio test to determine if the top model performed better than the reduced model. Additionally, the Wald Test confirmed each coefficient's significance in the model.

To assess how dwarf wedgemussel abundance (considered as CPUE) was related to host fish abundance, I used a mixed effects Tweedie Generalized Linear Model (GLM) (Tweedie 1984) using the 'cpglmm' function in the 'cpglm' package in R (Dunn and Smyth 2005). The mixed effects Tweedie GLM is a flexible regression model that is

useful for handling catch-per-unit-effort response variables with a high number of zeros (Shono 2008, Forrestal *et al.* 2019) The appropriate variance power for each analysis was determined using maximum likelihood estimations (MLE) (Dunn and Smyth 2008, Hasan and Dunn 2011). I ran analyses for both watersheds in combination (with watershed as a random effect) and then ran a separate analysis for the Delaware River watershed (with stream as a random effect). For the analysis considering both watersheds, tessellated darter abundance was the only predictor tested because it was the only host fish collected from both watersheds. For the analysis in the Delaware watershed, all four host fish were included in the full model. To assess the final models for each dataset, each variable was analyzed using deviance tables and a corrected delta AIC for a small sample size. Models within 2 delta AIC units of each other were considered similar. All analyses were conducted in R-version 3.6.3 (R Core Team 2020).

3.3 Results

3.3.1 Fish Assemblages

While there were 2,291 point locations with dwarf wedgemussel data, only 65 of the locations were within 500 m of a fish survey location. The 65 sites were comprised of 45 dwarf wedgemussel presence locations and 20 absence locations from 8 waterbodies in the Connecticut River watershed (n=36 sites: 1 absence, 35 presence) and 5 waterbodies in the Delaware River watershed (n=29 sites: 19 absence, 10 presence) (Table 3.2).

Of the 46 fish species that were collected near dwarf wedgemussel sites, 25 species were found in both watersheds (Table 3.3); 9 species were unique to the

Connecticut watershed and 12 were unique to the Delaware watershed. Host fish species (brown trout, banded killifish, slimy sculpin, shield darter and tessellated darter) comprised 2,709 of the 10,277 fish collected (Table 3.1). Brown trout, slimy sculpin and banded killifish were only found at 1 site in the Connecticut, and no shield darters were found in the Connecticut River watershed. Additionally, no banded killifish were found in the Delaware River watershed. Only 5 fish surveys did not contain any host fish, and all of these were found near presence sites.

In the Delaware River watershed, total fish richness, total fish abundance and host fish abundance were higher in areas with dwarf wedgemussel present (vs. absent) (Figure 3.2). Total fish abundance was the only variable that was significantly higher near dwarf wedgemussel present (vs. absent) sites ($n = 29$, $F = 6.19$, $p = 0.019$) (Figure 3.4A). Tessellated darter abundance was also higher in sites with dwarf wedgemussel present ($\mu = 0.033$ CPUE) compared to absent ($\mu = 0.007$ CPUE) in the Delaware River watershed ($n = 29$, $p=0.004$; Figure 3.3). Because dwarf wedgemussel locations lacked absence locations in the Connecticut River watershed, I could not conduct independent analyses in this watershed.

Differences in fish assemblages were assessed using a 2-dimensional NMDS of all 65 fish surveys and produced a stress level of 0.19. The Connecticut and Delaware watersheds were dissimilar in their fish assemblages (Figure 3.4B) ($r^2 = 0.17$, $p < 0.001$). Dwarf wedgemussel presence and absence locations were also dissimilar in their fish assemblages (Figure 3.4B) ($r^2 = 0.10$, $p < 0.001$). The top 3 statistically significant, influential species driving the pairwise comparison differences between all dwarf wedgemussel occurrence locations were: white sucker (9.6%), fallfish (9.2%) and

blacknose dace (9.1%) (Table 3.4). A separate NMDS assessed the difference between dwarf wedgemussel presence and absence locations in the Delaware River watershed (n=29), however, there was no significant dissimilarity in fish communities found between groups (stress = 0.17, $r^2 = 0.05$, $p = 0.171$) (Figure 3.5).

3.3.2 Predictors of Dwarf Wedgemussel Presence and Abundance

The top GLMM model predicting the probability of a dwarf wedgemussel occurrence in both watersheds (n=65) included 2 fixed effects: tessellated darter abundance and brown trout abundance and a random effect of watershed (Table 3.6). Tessellated darter abundance was positively associated with dwarf wedgemussel presence and brown trout abundance was negatively associated with the dwarf wedgemussel presence (Figure 3.6). The univariate model that only included brown trout abundance as a predictor was equally plausible (i.e., ΔAICc < 2; Table 3.6). In the Delaware River watershed the top GLMM model predicting dwarf wedgemussel occurrence included a fixed effect of brown trout abundance and a random effect of stream (Table 3.5). Brown trout abundance was negatively related to dwarf wedgemussel occurrence (Figure 3.7). Other models that were equally plausible included tessellated darter and shield darter (i.e. < 2 ΔAICc; Table 3.5), which were both positively related to dwarf wedgemussel occurrence.

The top Tweedie GLMM model for predicting dwarf wedgemussel abundance in both watersheds included 1 fixed effect, tessellated darter abundance ($p < 0.001$) and a random effect of watershed (Table 3.7; Table 3.8). Tessellated darter abundance was positively associated to dwarf wedgemussel abundance. In an additional analysis that

only included fish surveys from the Delaware watershed, the top model included 2 fixed effects: brown trout abundance (-1969.04, $p = 0.002$) and tessellated darter abundance (30.64, $p < 0.001$) as well as a random effect of stream (Table 3.1). Other models were considered, but none were equally as plausible (e.g. < 2 ΔAICc; Table 3.7).

3.4 Discussion

This is the first study to explore host fish as they relate to dwarf wedgemussel abundance and occurrence in the wild across multiple watersheds. Overall fish abundance was higher at dwarf wedgemussel presence versus absence sites in the Delaware River watershed suggesting that fish abundance plays an important role in dwarf wedgemussel reproduction. Fish assemblages also differed between dwarf wedgemussel present versus absent locations; however, this is likely due to the difference in sampling efforts between the 2 watersheds. Two fishes that transformed dwarf wedgemussel in laboratory experiments (St. John White *et al.* 2017) emerged as significant predictors of dwarf wedgemussel abundance. Tessellated darter abundance had a positive relationship to dwarf wedgemussel abundance and occurrence across both watersheds, while brown trout abundance negatively correlated to dwarf wedgemussel abundance. Slimy sculpin, which transformed the highest proportion of mussels in the lab (transformation = 90%) (St. John White *et al.* 2017), and shield darter (transformation = 16%) did not emerge as significant predictors in any analyses.

3.4.1 Fishes Near Dwarf Wedgemussel Sites

Total fish richness and host fish richness appeared higher in dwarf wedgemussel presence vs absence locations, but no significant difference was found. The lack of difference in fish richness (host or otherwise) may highlight that richness may not be an important influence for dwarf wedgemussel presence in the wild, or this finding may be a result of low sample size. This finding contradicts other studies that found host fish richness was an important predictor for other *Alasmidonta* species such as the elktoe and slippershell (Daniel *et al.* 2018). Host fish richness may be an important predictor for measuring mussel habitat suitability, as mussels and their corresponding host fish likely occupy similar habitat (Daniel *et al.* 2018). However, because host fish requirements for the dwarf wedgemussel are not entirely known, total fish richness likely offers valuable information for understanding reproductive potential for dwarf wedgemussel.

Total fish abundance was higher in dwarf wedgemussel presence locations than absence locations in the Delaware River watershed, suggesting that fish abundance may influence dwarf wedgemussel occurrence. Fish abundance is especially important for reproductive success in a mussel that utilizes small-bodied host fish, like the dwarf wedgemussel. Larger-bodied fish, such as salmonids, can maintain high infestation rates of glochidia, with infection rates as high as 250 glochidia per fish (Ieshko *et al.* 2016). Small-bodied fish, on the other hand, generally have lower infestation rates. In one study in Massachusetts, 67% of infected tessellated darter only carried 1 glochidia, with only a maximum of 3 glochidia found on each fish throughout the study (McLain and Ross 2005). St. John White's (2007) investigation of the Ashuelot River in New Hampshire found a total of 26 glochidia on a single tessellated darter in the wild. These findings

support the idea that small-bodied host fish may be required in larger abundances to promote successful reproduction.

Fish assemblages varied between dwarf wedgemussel presence versus absence locations. However, the differences in assemblages may also be explained by differences in watersheds, as an artifact of skewed sampling effort in the 2 watersheds. The Delaware River watershed included more fish surveys near absence locations because it was systematically surveyed for dwarf wedgemussels and thus generated a high number of absence locations near fish surveys. In contrast, fish surveys from the Connecticut River watershed were not randomly selected and were generally situated near dwarf wedgemussel presence locations (presence = 35, absence = 1). Furthermore, because the Connecticut River watershed only contained 1 dwarf wedgemussel absence location, and only 5 locations with abundance data, the Connecticut River watershed did not contain enough data to warrant its own analysis. A relationship may emerge if additional fish sampling effort near dwarf wedgemussel absence locations can be completed in the Connecticut River watershed.

Tessellated darter was the only host fish to emerge as a significant, positive predictor of dwarf wedgemussel. This was not a surprise, considering dwarf wedgemussel and tessellated darter occupy similar habitats in the Delaware and Connecticut River watersheds. The tesssellated darter is a habitat generalist and tends to occupy sandy substrates in areas with low to moderate flow (Carleson 2008), much like the dwarf wedgemussel (Galbraith *et al.* 2016). The tessellated darter is also a benthic feeder (Morissette *et al.* 2018) that primarily relies on a diet of chironomid larvae in the

substrate (Layzer and Reed 1978), which would often put tessellated darter in close proximity to gravid dwarf wedgemussels.

Interestingly, brown trout, which was a potential host fish in the lab (St. John White *et al.* 2017), was negatively related to dwarf wedgemussel presence. It is possible that brown trout actively prey on other host fish for dwarf wedgemussel, such as tessellated darter and sculpin, explaining the negative relationship. Native sculpin and other small fish have commonly been found in the stomachs of brown trout (Meredith *et al.* 2016). Another possible explanation of the negative relationship is that brown trout occupy different habitats than the dwarf wedgemussel, and thus the relationship with dwarf wedgemussel is explained by habitat preferences. Brown trout often prefer medium to coarse substrates and tend to occupy pools in streams (Ayllón *et al.* 2010) and move into wider and deeper sections of river as they grow. Brown trout also prefer 50% or more plant cover to protect themselves from predators (Armstrong *et al.* 2003) and they feed in littoral areas (Lobón-Cerviá and Sanz 2017). In contrast, dwarf wedgemussel are often found in shallow areas with sandy substrates and low plant cover in the Delaware River watershed (Galbraith *et al.* 2016). Difference in habitat preference, indicate that the brown trout are not a likely host fish for dwarf wedgemussel in the wild.

3.4.2 Potential Host Fish for Dwarf Wedgemussel

As mentioned earlier, dwarf wedgemussel glochidia have been observed on the bodies of tessellated darter in the wild (Michaelson and Neves 1995, McLain and Ross 2005, Wicklow 2004). Observations in the wild in conjunction with similar habit use to dwarf wedgemussel and high transformation in laboratory results (St. John White *et al.*

2017) all support tessellated darter as an important host fish for dwarf wedgemussel in the wild.

Two additional fishes that transformed dwarf wedgemussel glochidia in the lab, slimy sculpin and shield darter, also appeared in Delaware River watershed fish surveys; however, neither of these species emerged as top predictors for dwarf wedgemussel abundance or occurrence in any analysis. St. John White (2007) noted that shield darter co-occurred with dwarf wedgemussel locations in the Delaware River watershed over several years. In the surveys, shield darter appeared in 34.5% of sites in the Delaware River watershed; however, the species appeared in low abundances (1.46% relative abundance). The difference between St. John White's (2007) observations and my findings may have been due to the small home ranges that sculpins and darters have (<10m) (McLain and Ross 2005, Keeler *et al.* 2007). Because fish surveys could have been up to 500 m from dwarf wedgemussel populations, it is possible that shield darter may only be found very close to dwarf wedgemussel. The lack of relationship may have also been due to low abundance of shield darter. Slimy sculpin were also of particular interest in this study because it transformed the highest proportion of dwarf wedgemussel glochidia in the lab (transformation = 90%) (St. John White *et al.* 2017). However, in St. John White's (2007) surveys it was noted that slimy sculpin rarely co-occurred with dwarf wedgemussel. In my study, slimy sculpin were found in 37.9% of sites in the Delaware River watershed in reasonably high abundances (9.04% relative abundance). Their lack of significance in analyses could have been the result of a small sample size, and additional fish surveys with different fish collection methods more suitable to

collecting benthic fishes may produce a significant relationship between dwarf wedgemussels and slimy sculpin.

Although it was beyond the scope of this study, to learn more about dwarf wedgemussel host fish in the wild, future studies could conduct field surveys in areas with dense dwarf wedgemussel populations, when the mussels are releasing their glochidia (in April to June) and inspect all fishes for attached glochidia. This would expand upon the study conducted by McLain and Ross (2005), who collected tessellated darter in a 50-m section of river from May until June and inspected their gills for encystment of glochidia. By inspecting more fish species in more streams in the wild, researchers could gain a better understanding of what host fish are utilized in the wild. These projects should be done with great care and densities of the local dwarf wedgemussel populations should be well understood to avoid trampling dwarf wedgemussels in dense populations. Also surveying and inspecting fish while mussels are releasing their glochidia may cause undue stress on fishes and could cause harm to the glochidia, so these tradeoffs should be considered.

3.4.3 Fish Considerations for Conservation of Dwarf Wedgemussel

Propagation and restoration of freshwater mussels has become more a feasible conservation strategy due to improved laboratory techniques. Researchers can now produce freshwater mussels by the thousands (Patterson *et al.* 2018). Now, because of high production, it is important to develop clear restoration plans to ensure propagated individuals survive in the wild (Strayer *et al.* 2019). Before propagated mussels can be released, actions need to be taken to ensure mussels' long-term survival and proliferation

in the wild. These actions include improving habitat quality and improving and maintaining water quality (Nakamura 2019). If the mussels are being returned to an area where there was a known historic population, it is important to confirm that the threats that may have caused the decline of the species have been mitigated (Strayer *et al*. 2019). Determining abundances of potential host fish at possible reintroduction sites is an important step in selecting locations for reintroducing or augmenting for propagated or translocated mussels (McMurray and Roe 2017).

Conservation efforts for dwarf wedgemussels should also include conservation efforts for native host fish species. The protection of native host fishes will vary widely depending on geographic location because of potential differences in host fish across their range; however, the creation and maintenance of diverse microhabitats may promote a rich, native fish community and should be a focus of their management. A diverse habitat contains a variety of substrate types and sizes, along with wood (Allan 2004, Wheeler *et al.* 2005). Channelized streams also negatively influence native fish communities as they reduce habitat diversity, stability and eliminates pool – riffle sequences in the stream (Wheeler *et al.* 2005). Other considerations for preserving healthy native fish communities include protection of large riparian zones (Allan 2004), which offer cover for fish and filter sediment and nutrient runoff from surrounding land (Wheeler *et al.* 2005). Habitat fragmentation caused by dams is another critical concern for native fish communities (Daniel *et al.* 2018). Dams not only isolate mussel and fish populations from one another, but they also severely alter surrounding downstream habitat. A dam can alter flow, temperature, dissolved oxygen, and substrate downstream of the dam, making it unsuitable for certain native fishes (Daniel *et al.* 2018). Finally,

ongoing stream management at potential restoration locations should include the needs of sensitive, native species like darters and sculpin (Adams and Schmetterling 2007). Overall, conservation and management goals may be met better by protecting the habitat of small-bodied fish like sculpin and darters that have very small home ranges (McLain and Ross 2005, Keeler *et al.* 2007) which makes them sensitive to environmental changes (Adams and Schmetterling 2007).

3.5 Conclusion

Dwarf wedgemussels are at risk for becoming extinct due to low densities, small ranges, and population patchiness (Galbraith *et al.* 2016). Through the development of comprehensive restoration plans that include mussels as well as their potential host fish we can better protect their future decline. By focusing on the protection of tessellated darter with consideration of other native fish species, we may offer better protection for dwarf wedgemussel populations, since their host-specificity is still largely unknown. However, the influence of host fish on dwarf wedgemussel is just one of many steps for successful restoration. Information on water quality and habitat (McMurray and Roe 2017), as well as proximate factors that may alter these conditions, is critical prior to augmentation or re-introduction. By managing potential dwarf wedgemussel restoration locations holistically, with the consideration for abiotic and biotic factors, we can better protect the entire stream ecosystem (Fritts *et al.* 2012). Through connected management we can select and protect restoration locations for freshwater mussels and bring us closer to the preservation of dwarf wedgemussel populations.

Table 3.1. Fish species with successful lab transformation of glochidia (for dwarf wedgemussel St. John White *et al.* 2017) and the relative abundance and numbers of sites (of 65) where the fish species was observed near dwarf wedgemussel locations in the Connecticut (C) and Delaware (D) River watersheds.

Common name	Scientific name	Transformation success	Relative abundance	Sites observed	Watershed
Slimy sculpin	*Cottus cognatus*	0.90	649	12	C, D
Striped bass	*Morone saxatilis*	0.79	0	0	D
Tessellated darter	*Etheostoma olmstedi*	0.44	1430	57	C, D
Mottled sculpin	*Cottus bairdii*	0.41	0	0	D
Atlantic salmon	*Salmo salar*	0.23	0	0	C, D
Shield darter	*Percina peltate*	0.16	105	10	D
Brown trout	*Salmo trutta*	0.14	455	19	C, D
Banded killifish	*Fundulus diaphanus*	0.08	10	1	D

Table 3.2. Stream locations of dwarf wedgemussel presence (n=45) and absence (n=20) locations in the Connecticut (n = 36) and Delaware (n =29) River watersheds.

Stream	State	Watershed	Presence Sites	Absence Sites	Total sites
Ashuelot River	New Hampshire	Connecticut	3	0	3
Broad Brook	Massachusetts	Connecticut	1	0	1
Connecticut River	New Hampshire	Connecticut	20	1	21
Fort River	Massachusetts	Connecticut	3	0	3
Mill River	Massachusetts	Connecticut	5	0	5
Muddy Brook	Connecticut	Connecticut	1	0	1
Philo Brook	Connecticut	Connecticut	1	0	1
Stony Brook	Connecticut	Connecticut	1	0	1
Little Flat Brook	New Jersey	Delaware	2	1	3
Paulinskill	New Jersey	Delaware	3	3	6
Flat Brook	New Jersey	Delaware	3	2	5
Neversink River	New York	Delaware	2	13	15

Table 3.3. All fish species found in the 65 sites in the Connecticut (n=36) and Delaware (n=29) River watersheds, ordered by percent relative abundance.

Common name	Scientific name	Count	Relative abundance (%)	Sites found	Sites where fish occurred (%)
Tessellated darter	*Etheostoma olmstedi*	1430	13.91	57	87.7
White perch	*Morone americana*	1219	11.86	45	69.2
Fallfish	*Semotilus corporalis*	975	9.49	40	61.5
Blacknose dace	*Rhinichthys atratulus*	941	9.16	30	46.2
Slimy sculpin	*Cottus cognatus*	649	6.32	12	18.5
Common shiner	*Luxilus cornutus*	579	5.63	27	41.5
American eel	*Anguilla rostrata*	523	5.09	28	43.1
Cutlips minnow	*Exoglossum maxillingua*	469	4.56	15	23.1
Longnose dace	*Rhinichthys cataractae*	460	4.48	27	41.5
Brown trout	*Salmo trutta*	455	4.43	19	29.2
Creek chub	*Semotilus atromaculatus*	356	3.46	18	27.7
Smallmouth bass	*Micropterus dolomieu*	266	2.59	30	46.2
Brook trout	*Salvelinus fontinalis*	237	2.31	12	18.5
Redbreast sunfish	*Lepomis auritus*	216	2.10	16	24.6
Redfin pickerel	*Esox americanus*	178	1.73	12	18.5
Spotfin shiner	*Cyprinella spiloptera*	158	1.54	15	23.1
Rock bass	*Ambloplites rupestris*	152	1.48	24	36.9
Sea lamprey	*Petromyzon marinus*	149	1.45	15	23.1
Margined madtom	*Noturus insignis*	116	1.13	12	18.5
Shield darter	*Percina peltata*	105	1.02	10	15.4
Largemouth bass	*Micropterus salmoides*	100	0.97	21	32.3
Yellow perch	*Perca flavescens*	76	0.74	19	29.2
Rainbow trout	*Oncorhynchus mykiss*	62	0.60	9	13.9
Yellow bullhead	*Ameiurus natalis*	59	0.57	12	18.5
Chain pickerel	*Esox niger*	58	0.56	16	24.6
Pumpkinseed	*Lepomis gibbosus*	50	0.49	21	32.3
Green sunfish	*Lepomis cyanellus*	48	0.47	11	16.9
Eastern mudminnow	*Umbra pygmaea*	43	0.42	6	9.2
Spottail shiner	*Notropis hudsonius*	38	0.37	1	1.5
Bluegill	*Lepomis macrochirus*	36	0.35	16	24.6
Stonecat	*Noturus flavus*	13	0.13	3	4.6
Walleye	*Sander vitreus*	12	0.12	5	7.7
Central mudminnow	*Umbra limi*	11	0.11	7	10.8
Brown bullhead	*Ameiurus nebulosus*	10	0.10	10	15.4
Burbot	*Lota lota*	6	0.06	1	1.5

Northern hogsucker	*Hypentelium nigricans*	6	0.06	2	3.1
Creek chubsucker	*Erimyzon oblongus*	4	0.04	1	1.5
Bluntnose minnow	*Pimephales notatus*	2	0.02	1	1.5
Northern pike	*Esox lucius*	2	0.02	2	3.1
Black crappie	*Pomoxis nigromaculatus*	2	0.02	1	1.5
Goldfish	*Carassius auratus*	1	0.01	1	1.5
Lake chub	*Couesius plumbeus*	1	0.01	1	1.5
Bridled shiner	*Notropis bifrenatus*	1	0.01	1	1.5
Banded killifish	*Fundulus diaphanus*	1	0.01	1	1.5
Bluespotted sunfish	*Enneacanthus gloriosus*	1	0.01	1	1.5
Satinfish shiner	*Cyprinella analostana*	1	0.01	1	1.5

Table 3.4. Fish species that are the driving contributors (>3%) distinguishing dwarf wedgemussel presence and absence locations in the Connecticut and Delaware River watersheds in the Nonmetric Multidimensional Scaling (NMDS) ordination. All p-values were calculated with false discovery rate (FDR) p-value. Glochidia transformation success proportions based on St. John White *et al.* (2017) included, where 0 means no glochidia transformed, but the fish were tested and NA means the fish was not tested. Bold font indicates significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Common name	Scientific name	Contribution (%)	FDR p-value		Transformation success
White sucker	*Catostomus commersonii*	9.6	**0.022**	*	0
Tessellated darter	*Etheistoma olmstedi*	9.5	0.423		0.44
Fallfish	*Semotilus corporalis*	9.2	< **0.001**	***	NA
Blacknose dace	*Rhinichthys atratulus*	9.1	**0.034**	*	0
Brown trout	*Salmo trutta*	8.1	< **0.001**	***	0.14
Slimy sculpin	*Cottus cognatus*	8.0	**0.011**	*	0.90
Common shiner	*Luxilus cornutus*	5.2	0.850		0
Cutlips minnow	*Exoglossum maxillingua*	5.1	0.080		0
American eel	*Anguilla rostrata*	4.5	**0.014**	*	0
Longnose dace	*Rhinichthys cataractae*	3.5	**0.007**	**	0

Table 3.5. Model selection for dwarf wedgemussel occurrence using mixed effects generalized linear model with a binomial distribution and a complementary log-log link transformation. Results and rankings for all models tested explaining occurrence of dwarf wedgemussel locations in the Connecticut River and Delaware River watersheds include the random effect of "watershed." All models explaining occurrence of dwarf wedgemussel locations in the Delaware River watershed include the random effect of "stream". All host fish were standardized with catch-per-unit-effort (shock seconds). *K* = number of parameters, AIC_c = Akaike Information Criterion with a correction for small sample sizes, and LL = log likelihood.

All Models	*K*	AIC_c	ΔAIC_c	Weights	LL
Both watersheds					
Tessellated darter + Brown trout	4	49.00	0.00	0.52	-20.17
Brown trout	3	49.62	0.62	0.37	-21.62
Tessellated darter	3	52.29	3.29	0.10	-22.94
Null + (random effect)	2	57.99	8.99	0.01	-26.90
Delaware River watershed					
Brown trout	3	32.33	0.00	0.25	-13.16
Tessellated darter + Brown trout	4	33.67	1.34	0.20	-12.00
Brown trout + Shield darter	4	33.97	1.64	0.18	-12.15
Tessellated darter + Brown trout + Shield darter	5	35.17	2.84	0.09	-11.28
Tessellated darter + Brown trout + Slimy sculpin	5	36.35	4.02	0.05	-11.87
Tessellated darter	3	36.61	4.28	0.05	-14.83

Brown trout + Shield darter + Slimy sculpin	5	36.62	4.29	0.04	-12.00
Brown trout + Slimy sculpin	4	36.66	4.33	0.03	-13.00
Full	6	38.03	5.70	0.02	-11.11
Tessellated darter + Shield darter	4	38.27	5.94	0.02	-14.30
Shield darter	3	40.71	8.38	0.01	-16.87
Null (plus random effect)	2	41.26	8.93	0.01	-18.34
Slimy sculpin + Shield darter	4	43.16	10.83	0.01	-16.75
Slimy sculpin	3	43.54	11.21	0.01	-18.29

Table 3.6. Top models for assessing dwarf wedgemussel occurrence in the Connecticut and the Delaware River watersheds combined (n= 65). All models used a mixed effects binomial generalized linear model with a random effect of "watershed" and the Delaware River watershed (n=29) using a mixed effects binomial generalized linear model with a random effect of "stream." SE = standard error.

Effect	Estimate	SE	P
Both Watersheds			
Intercept	0.26	0.76	0.734
Brown trout	-457.92	356.18	0.199
Tessellated darter	21.51	12.89	0.095
Delaware River watershed			
Intercept	-0.03	0.38	0.93
Brown trout	-1290.00	984.10	0.19

Table 3.7. Model selection for dwarf wedgemussel abundance using mixed effects generalized linear model with a Tweedie distribution. Results and rankings for all models tested explaining abundance of dwarf wedgemussels in the Connecticut River (n=5) and Delaware River (n=27) watersheds combined included the random effect of "watershed." Model weights are derived from models from full predictor sets to the top model. Results and rankings for all models tested explaining abundance in the Delaware River watershed alone (n=27) included the random effect "stream." All fish were standardized using catch-per-unit-effort. *K* = number of parameters, AIC_c = Akaike Information Criterion with a correction for small sample sizes, and LL = log likelihood.

All Models	*K*	AIC_c	ΔAIC_c	Weights	LL
Both watersheds					
Tessellated darter	4	81.26	0.00	1.00	-35.89
Null + random effect	3	92.69	11.43	<0.01	-45.45
Delaware River watershed					
Tessellated darter + Brown trout	5	60.38	0.00	0.53	-23.76
Tessellated darter + Brown trout + Slimy sculpin	6	62.67	2.29	0.17	-23.23
Tessellated darter + Brown trout + Shield darter	6	63.42	3.04	0.12	-23.61
Tessellated darter	4	63.96	3.58	0.09	-27.07
Brown trout	4	66.24	5.86	0.03	-28.21
Slimy sculpin + Tessellated darter	5	66.24	5.86	0.03	-26.69
Brown trout + Shield darter	5	66.64	6.26	0.02	-26.89
Brown trout + Slimy sculpin	5	68.98	8.60	0.01	-28.06
Shield darter	4	71.01	10.63	<0.01	-30.60

Null (with random effect)	3	72.69	12.31	<0.01	-32.83
Slimy sculpin + Shield darter	5	73.92	13.54	<0.01	-30.53
Slimy sculpin	4	75.47	15.09	<0.01	-32.82

Table 3.8. Parameter estimates from top mixed effects Tweedie generalized linear model models for assessing dwarf wedgemussel abundance in the Connecticut and the Delaware River watersheds combined (n= 32). All models used a random effect of "watershed," and in the Delaware River watershed alone (n=27), which used a random effect of "stream." P-values were calculated using the likelihood ratio test for univariate models for each variable. SE = standard error.

Effect	Estimate	SE	t-value	p
Both watersheds				
Intercept	-1.14	0.40	-2.88	
Tessellated dater	40.05	7.30	5.48	<0.001
Delaware River watershed				
Intercept	-0.42	0.59	-0.71	
Brown trout	-1969.04	1441.06	-1.37	0.002
Tessellated darter	30.64	9.69	3.16	<0.001

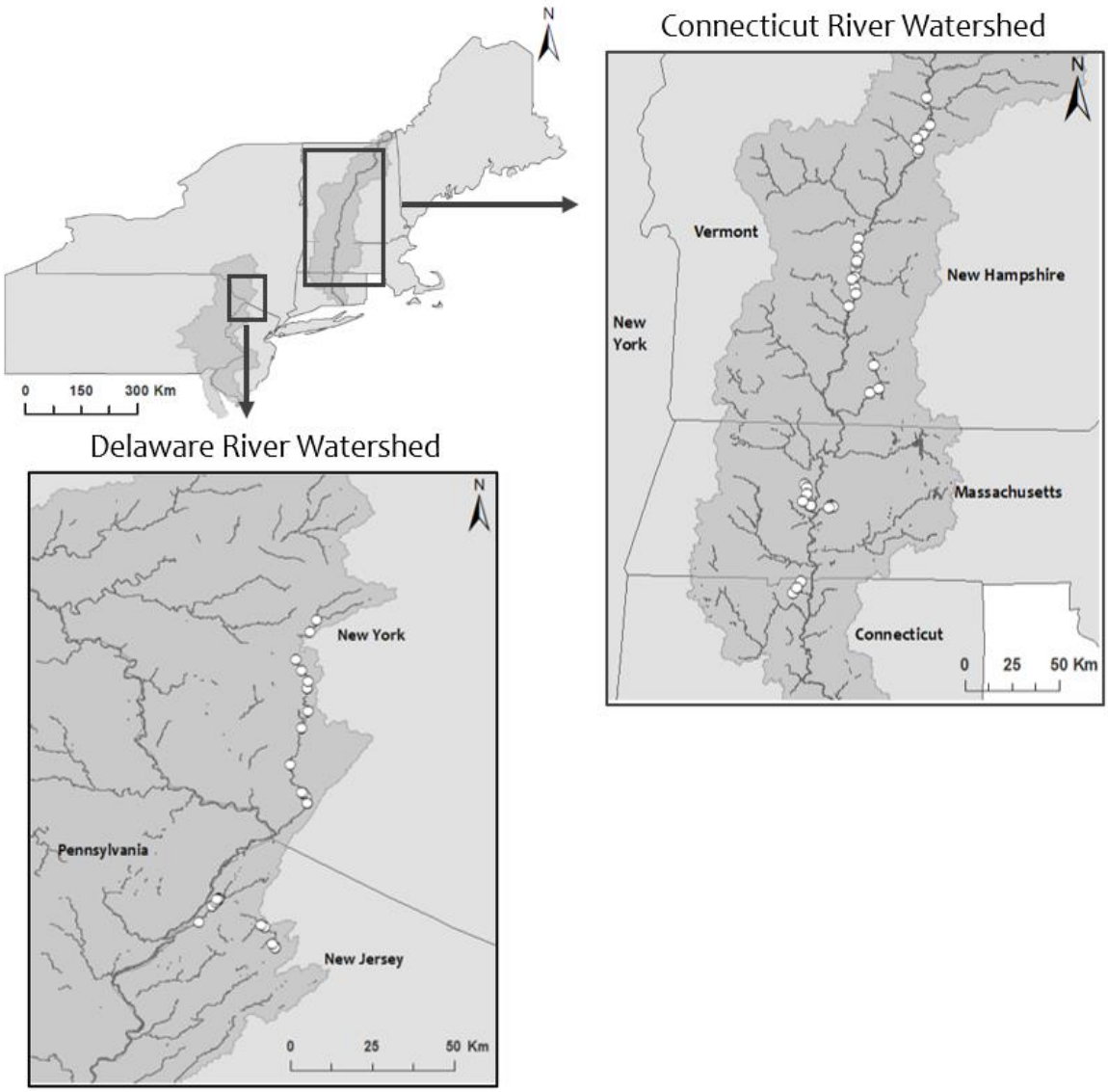


Figure 3.1. Map of fish survey sites near dwarf wedgemussel presence and absence locations in the Connecticut River (n = 36) and Delaware River (n = 29) watersheds in the northeastern US.

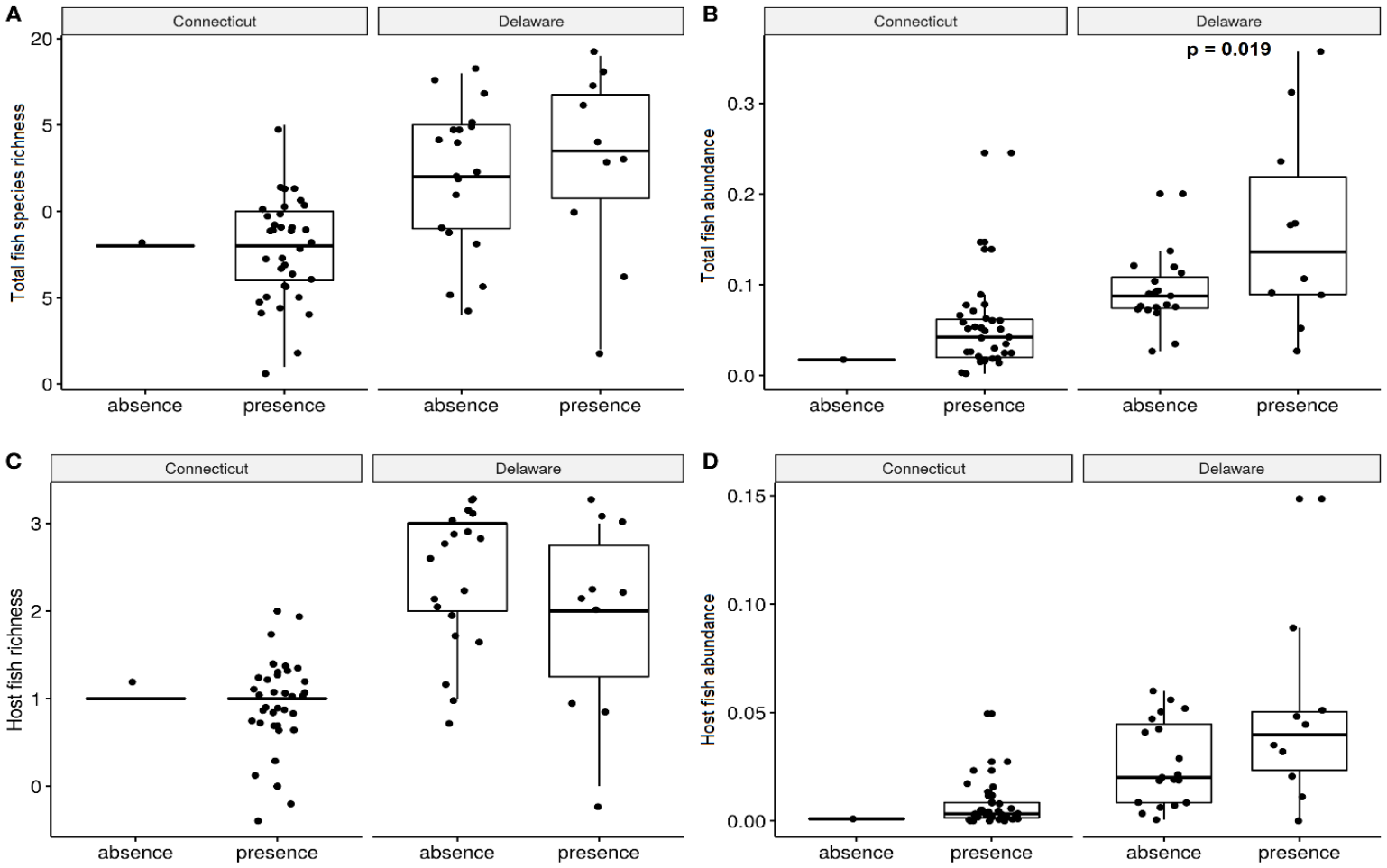


Figure 3.2 Boxplots (median and interquartile; whiskers = min and max value) of A) total fish richness, B) total fish abundance, C) host fish richness, and D) host fish abundance at dwarf wedgemussel presence and absence locations in the Connecticut (n= 36) and Delaware River (n=29) watersheds. P-value for one-way analysis of variance comparing presence vs absence within each watershed reported where there were significant differences ($p<0.05$).

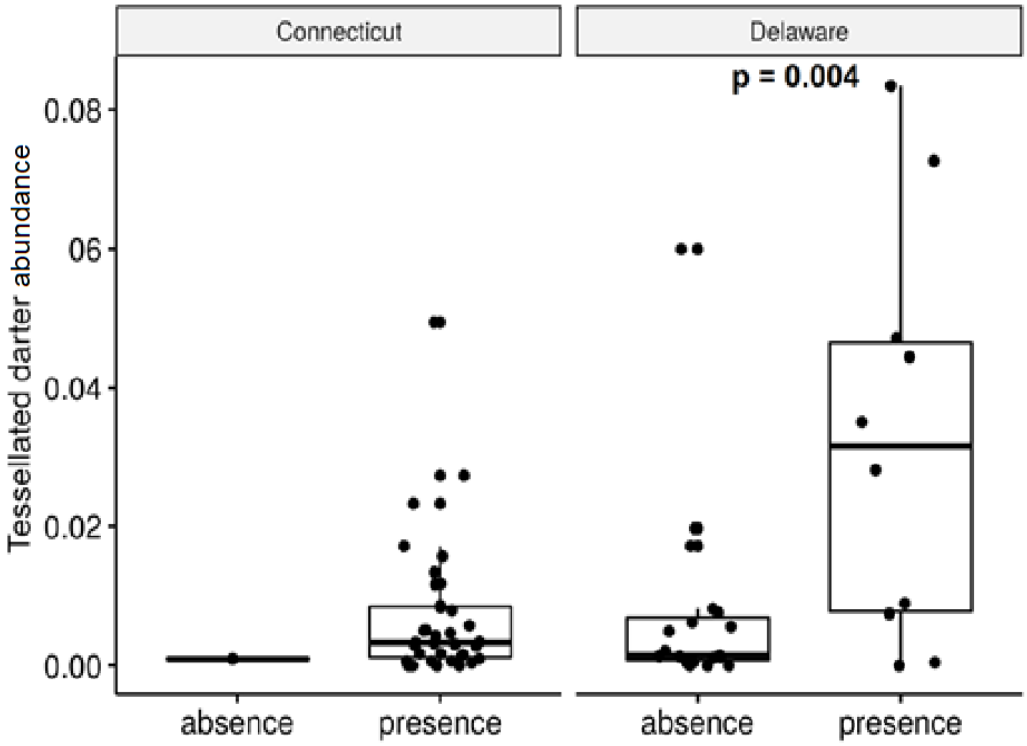


Figure 3.3. Tessellated darter abundance at dwarf wedgemussel presence and absence locations in the Connecticut River (n = 36 sites; F= 0.369 p = 0.548) and Delaware River (n = 29 sites; F= 10.01 p = 0.004) watersheds. Boxes represent median and interquartiles, and whiskers represent minimum and maximum values. p–values derived from one-way analysis of variance.

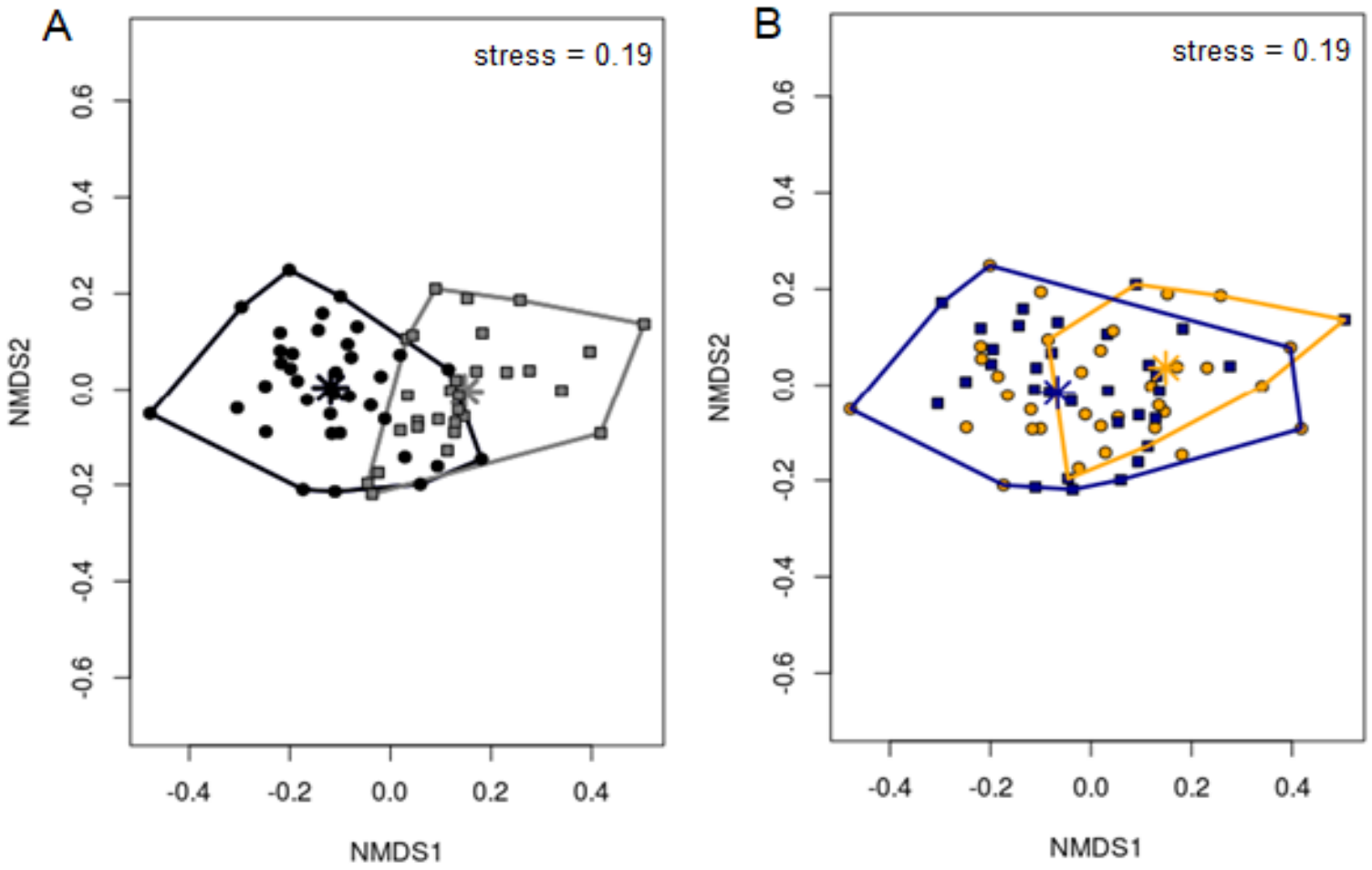


Figure 3.4. Nonmetric Multidimensional Scaling (NMDS) plot of the fish communities based on the Bray-Curtis distance metric. Sites are coded by A) the Connecticut River (grey squares; n = 36 sites) and Delaware River (black circles, n = 29 sites) watersheds locations with calculated centroids (R^2= 0.16, $p < 0.001$) and B) dwarf wedgemussel presence (blue squares, n = 45 sites) and absence (orange circles, n = 20 sites) locations with calculated centroids (R^2=0.10, $p < 0.001$).

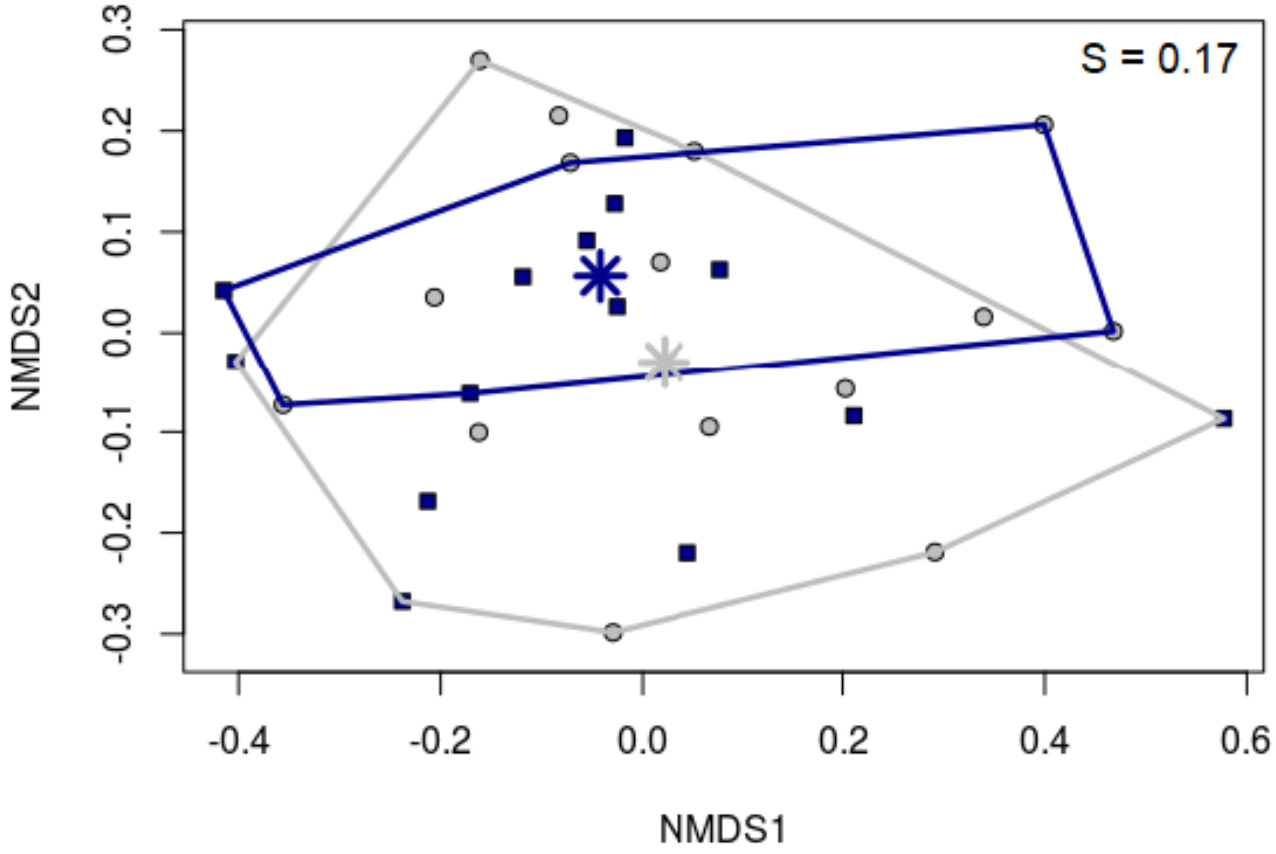


Figure 3.5. Nonmetric Multidimensional Scaling (NMDS) for presence/absence of dwarf wedgemussel in the Delaware watershed. Presence sites are represented in blue and absence sites are represented in grey. Multivariate analysis of variance: $R^2 = 0.05$, $p = 0.171$.

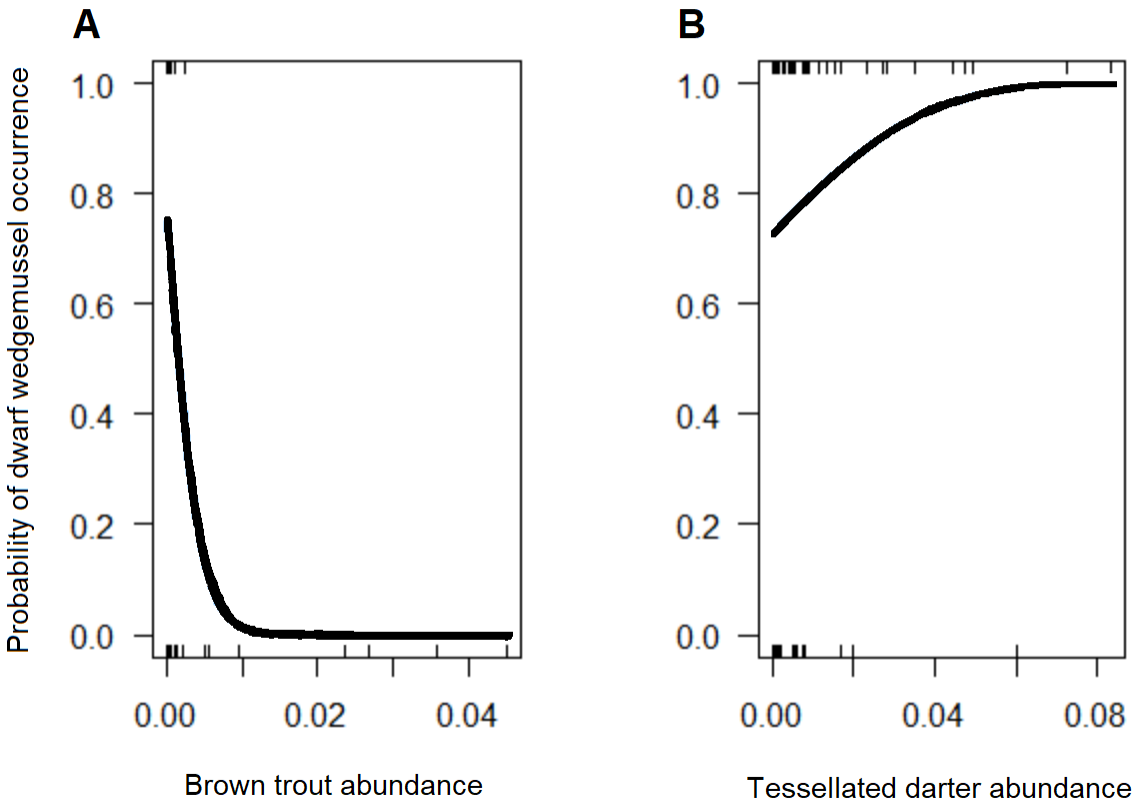


Figure 3.6. Model predictions for probability of dwarf wedgemussel occurrence based on A) brown trout abundance and B) tessellated darter abundance in both watersheds (n=34).

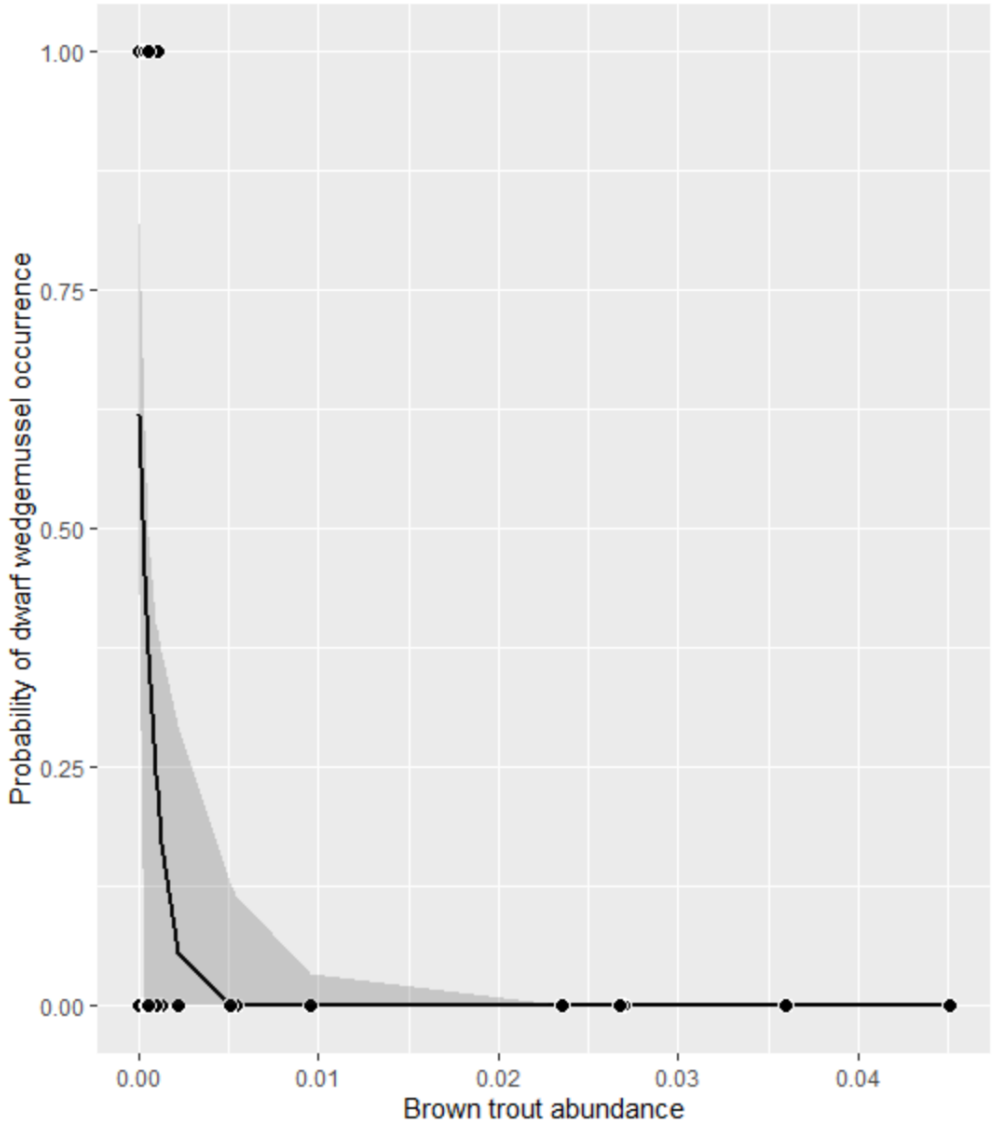


Figure 3.7. Model prediction for probability of dwarf wedgemussel occurrence based on brown trout abundance in the Delaware River watershed (n = 29, p = 0.19).

CHAPTER 4

CONCLUSION

Freshwater mussels (Unionidae) are a globally imperiled group of organisms that have declined in both distribution and abundance (Haag 2012). At first, declines were due to overharvesting in the late 1800s (Pritchard 2001), but the species has since declined due to habitat modification, water quality degradation, declines in host fish, introduction of invasive species and climate change (Ferreira-Rodríguez *et al.* 2019). Implementing effective management strategies to protect freshwater mussels is important and necessary for their conservation. Some management actions protect remaining viable mussel populations. For example, we can create riparian vegetative buffers around streams, open fish passages (Ferreira-Rodríguez *et al.* 2019), remove dams, and restore physical in-stream habitat to help conserve freshwater mussel populations and their hosts (Strayer *et al.* 2019). However, for some freshwater mussels species that are in critical low numbers and unlikely to recover in the wild, more extreme actions of propagation and re-introduction or augmentation may be needed to restore populations (Ferreira-Rodríguez *et al.* 2019). Propagation in combination with previously mentioned management actions could save the dwarf wedgemussel and other imperiled freshwater mussel species on the brink of becoming extinct.

This thesis contributes to data and methods needed to protect and recover dwarf wedgemussel, particularly in the northeastern portion of its range. The information collected will be used in conjunction with other research gathered on population viability, genetic and habitat assessments as a part of the U.S. Geological Survey Science Support

Partnership (SSP) with the U.S. Fish and Wildlife Service (USFWS) and add to the larger dwarf wedgemussel recovery plan (USFWS 1993). The SSP and recovery plan will be used to guide the recovery of dwarf wedgemussel through propagation and reintroduction to ultimately preserve the species and its habitat. My research contributed to these larger goals by developing *in-vitro* propagation methods for dwarf wedgemussel and methods for how to better mitigate contamination for higher transformation success. Additionally, through this research, I collected and compiled fish survey data throughout the Connecticut and Delaware River watersheds to assess abundances of potential host fish near current dwarf wedgemussel populations. Fish survey locations will be used in conjunction with habitat data to determine the best dwarf wedgemussel re-introduction locations.

In Chapter 2, I tested a variety of methods for contamination mitigation and how those methods affected transformation success of two *Alasmidonta* species, including the dwarf wedgemussel. Overall, I found that higher concentrations of the antifungal, Amphotericin B, did not appear to reduce contamination severity relative to lower concentrations and may relate to slightly lower transformation of glochidia. To get the highest transformation for *Alasmidonta* glochidia propagated *in-vitro*, I recommend using minimal (0–1 μg/mL) Amphotericin B in conjunction with a dose replenishment of the antifungal instead of frequent media changes, which may disrupt the developing glochidia (Lima *et al.* 2006). Additionally, I recommend that non-contaminated *in-vitro* dishes be opened infrequently to minimize exposure to contamination.

This study was the first to quantify the effect of contamination severity and its effect on glochidia transformation success. To assess and quantify contamination severity

is a procedure that could be adopted by other labs as a method to keep track of contamination and its impacts on glochidia across multiple species. Further investigation is needed to determine if the fungus found in my research is unique to the Richard Cronin Aquatic Resource Center (CARC) or if it is the same as the fungus impacting other *in-vitro* propagation facilities and experiments. By identifying the fungi plaguing *in-vitro* dishes across propagation facilities, we can use more targeted and specific treatment of fungal contaminants and potentially lead to more effective contamination mitigation (Ryan 1994). The fungi contaminating *in-vitro* dishes at CARC was *Candida parapsilosis*. Three echinocandin drugs are known to prohibit the growth and proliferation of *C. parapsilosis*: caspofungun, micafungin, and andulafungin (Tóth *et al.* 2019). Future studies should test to see how these drugs impact contamination and transformation success of glochidia. Quality *in-vitro* propagation methods for populations of dwarf wedgemussel will allow for more efficient production of a higher percentage of juveniles from glochidia (Lima 2012), which is critical for this species, given its low fecundity (Michaelson and Neves 1995). Developed and improved propagation methods can help protect the genetic variation within the species, which will promote the viability of the species as a whole. These methods can be used to help inform future propagation of dwarf wedgemussel, triangle floater, and potentially other *Alasmidonta* species, which will further conserve freshwater mussel species.

In Chapter 3, the positive correlation between tessellated darter abundance and dwarf wedgemussel presence and abundance supports the idea that tessellated darter are an important host fish species in the wild. This evidence is further reinforced by other research that have observed dwarf wedgemussel glochidia on the gills of tessellated

darter in the wild (McLain and Ross 2005). In this research we also found that there were distinct fish assemblages in dwarf wedgemussel presence versus absence locations in the Connecticut and Delaware River watersheds. Although differences may be a combined result of differences in sampling efforts and fish assemblages between watersheds, additional sampling of mussels and fish could tease out unique fish assemblages. Additionally, with more fish surveys comparing viable versus nonviable dwarf wedgemussel populations instead of presence versus absence locations, we may be able to determine which fishes are important for the persistence of viable populations, as we did not distinguish between viable and nonviable populations in our study. Through a better understanding of fish assemblages, densities, and potential host fishes near dwarf wedgemussel populations, researchers can place propagated juvenile dwarf wedgemussels at sites where they will have a greater chance of long-term persistence.

To date, dwarf wedgemussels have been extensively surveyed throughout its range to determine where viable populations of the species remain. Surveys conducted in conjunction with the dwarf wedgemussel recovery plan have documented 14 locations that currently hold viable populations: The Upper Connecticut River (NH/VT), Ashuelot River (NH), Mill River (MA), Fort River (MA), Muddy Brook (CT), Stony Brook (CT), Philo Brook (CT), Upper Delaware River (NY), Big/Little Flat Brook (NJ), Paulinskill (NJ), Nanjemoy Creek (MD), Tributary to Southeast Creek (MD), Po River (VA), Little Shocco Creek (NC), and Maple Branch (NC). Throughout the dwarf wedgemussel's historic range, other streams and rivers that once held dwarf wedgemussels are likely extirpated (n = 26), not viable (n=9) or are unknown (n=14) (USFWS 2019). Dwarf wedgemussel populations have declined in rivers like the Neversink in New York and the

mainstem of the Delaware River (New Jersey/Pennsylvania) due to flooding and drought (USFWS 2019), and the current viability of these populations is uncertain (USFWS 2019). In these areas of the Delaware River watershed, populations of dwarf wedgemussel are expected to decline or even disappear by the year 2050 as a result of minimal dispersal ability by the species combined with heavy rainfall, drought, and temperature changes due to climate change (Schledinger *et al.* 2011, Pennsylvania Natural Heritage Program 2011). While populations of dwarf wedgemussel in the Connecticut River watershed are expected to maintain their viability (USFWS 2019), southern populations in Virginia, North Carolina, and Maryland have predominantly low densities and reproductive rates. The outlook on dwarf wedgemussel recovery in their southern populations are not as positive compared to populations in the north, and the potential for recovery is low (USFWS 2019). However, the dwarf wedgemussel recovery plan laid out by the USFWS (1993) identifies strategies and programs to help conserve the species throughout its entire range.

The USFWS (1993) developed a recovery plan that identifies strategies for protecting the remaining dwarf wedgemussel populations and conserving the species. The plan also includes regular monitoring with reviews every 5 years to update information and action plans to reflect that information (Figure 4.1). As a part of the recovery plan, extensive surveys of dwarf wedgemussel populations have continued to identify new and persisting populations and assess their viability. Additionally, some fish hosts have been identified in the field (Michealson and Neves 1995, McLain and Ross 2005), and other potential fish hosts tested in the lab (St. John White *et al.* 2017). Successful propagation of dwarf wedgemussel has also been accomplished through my research at CARC, and at

the Aquatic Epidemiology and Conservation Lab at North Carolina State University (Michael Walter, personal communication). There have been extensive studies on dwarf wedgemussel habitat suitability (Maloney *et al.* 2012, Campbell and Hilderbrand 2016), flow preferences (Briggs *et al.* 2013, Parasiewicz *et al.* 2017), and thermal tolerances (Galbraith *et al.* 2020) to gain a better understanding of the species' habitat and stressors and thus, how to best protect it. Currently, under the SSP, genetic assessments of dwarf wedgemussel populations are being used to understand genetic differences among populations (Heather Galbraith, personal communication). Additionally, the SSP aims to locate potential sites for re-introduction and augmentation based on habitat, water quality, density of populations and host fish densities. In the future, additional priorities are still needed to conserve dwarf wedgemussel, including: additional population surveys (including eDNA surveys to help target survey locations), development of habitat protection strategies for declining populations, and development of methods to augment and eventually reintroduce dwarf wedgemussels (USFWS 2019). All of this research will help to better protect remaining populations and restore declining populations toward effective recovery of the dwarf wedgemussel.

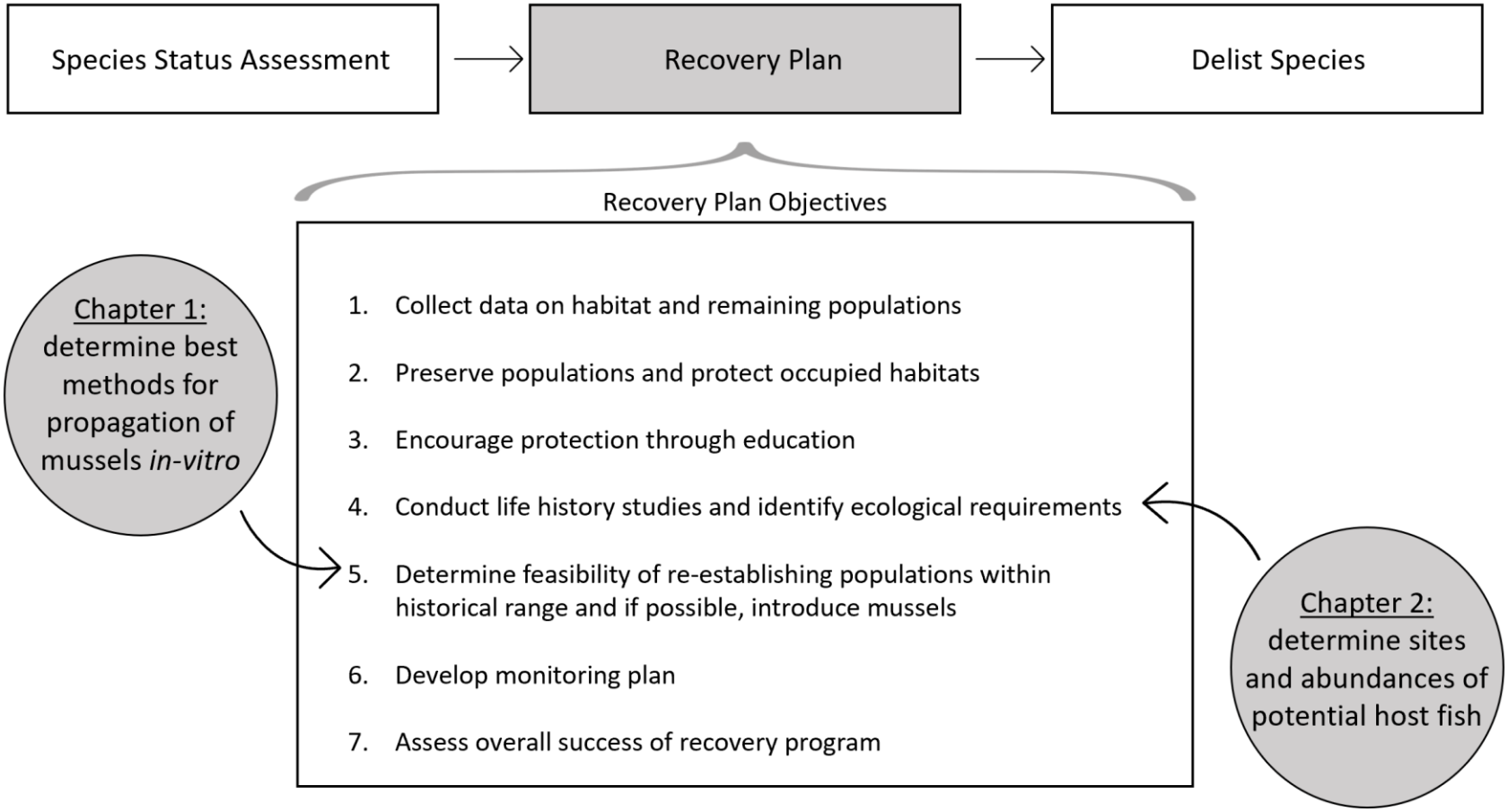


Figure 4.1 Conceptual model of the dwarf wedgemussel recovery plan process and how the research in this thesis has supported the plan in meeting its objectives (USFWS 1993).

APPENDICES

APPENDIX A

STANDARD OPERATING PROCEDURE FOR DNA EXTRACTION FOR FUNGI, OOMYCETES, AND BACTERIA

Prepared by: _____

Claire Walsh Date

Reviewed by: _____

Dr. Robert Wick Date

_____

Jennifer Ryan Date

1.0 Scope and Application

1.1 The purpose of this Standard Operating Procedure (SOP) is to describe the materials, standard methods, and quality assurance/quality control (QA/QC) measures used in the DNA extraction of fungi, oomycetes, and bacteria. Following the steps and methods described in this document will promote consistent techniques for DNA extraction.

1.2 The data collected through this SOP was used to layout techniques for DNA extraction of fungi contaminants found in *in-vitro* propagation dishes in triangle floater (*Alasmidonta heterodon*) culture.

2.0 Method Summary

Methods for DNA extraction is partially modeled after Wick *et al.* (1997) who extracted DNA from species such as *Phytophthora drechsleri, Pythium*

irregulare, *P. sylvaticum,* and *Rhizoctonia solani*. All lab equipment should be inspected for damage and repaired (as needed) to ensure consistent DNA extraction techniques. Before starting any protocol, all materials and workspaces should be wiped down using gloves and 70% ethanol. All tubes and samples should clearly be labeled with a name and a date.

Methods for DNA extraction may vary on the species and the most reliable method of DNA extraction is using a kit such as the Qiagen DNeasy Plant Mini Kit or boiling at 45℃ for 20 minutes (Wick *et al.* 1997). You can also extract directly into a prepared PCR mix. For very difficult to obtain DNA, lyophilize (freeze dry) a section of the sample before using the Qiagen DNeasy Plant Mini Kit.

3.0 Definitions and Abbreviations

Buffers (AP1, AE, AW1, AW2, P3)	Maintains the integrity of the DNA during extraction, and separates DNA from other cell debris. All buffers mentioned were provided in Qiagen DNeasy Plant Mini Kit
DNA	deoxyribonucleic acid, carries genetic information
Isolate	Isloated DNA sample
Lyophilize	Process of freeze drying a sample
Lyses	Process of rupturing cell walls
mycelium	Vegetative network of a fungus or fungus-like organism, consisting of branching, thread-like hyphae, that proliferates on agar

PCR	Polymerase chain reaction, a process used to make copies of a DNA sample
PCR Master mix	Batch of PCR reagents optimized for DNA extraction that contain buffers and primers
RNase A	Ribonuclease A, an enzyme used for DNA purification
supernatant	Liquid above a solid residue that forms after centrifuging

4.0 Health and Safety Concerns

Ethanol (70%) will be used for sterilizing materials and work spaces. Ethanol is a Class 1B flammable liquid and requires handling and storage consistent with local fire codes. All USGS Cooperative Unit and UMass Amherst members must also complete lab and fire safety training at UMass Amherst.

5.0 Personal Qualifications

The person tasked with DNA extraction should have sufficient experience handling fungal, oomycete or bacteria samples and with standard PCR methods. This person must be experienced enough to provide guidance and instruction for less experienced assistants.

6.0 Materials and Supplies

- Nitrile sterile gloves
- Centrifuge with temperature control
- 70% Ethanol
- Aluminum inoculation loop

- Incubator
- Thermal beans or crushed ice
- Fume hood or Biosafety Cabinet
- Water bath
- Fungal or oomycete isolate in solid or liquid medium
- Sterile inoculation loop or agar-cutting tool, as needed
- Sterile 2mL tubes or PCR tubes containing master mix
- Thermocycler
- QIA shredder mini spin column tubes
- Qiagen DNeasy Plant Mini Kit and its supplies
- Nanodrop Microvolume Spectrophotometer
- P-10, P-200 and P-1000 pipette and tips
- Sterile 2 mL Tubes
- Microcentrifuge or vortex for 2 mL tubes
- Water bath with controlled temperature
- Ice Bucket with Ice or Cool Beans
- Nanodrop and sterile water

7.0 **DNA Extraction using Qiagen DNeasy Plant Mini Kit Method**

1. Turn on a fume hood or biosafety cabinet and clean all surfaces with ethanol, including gloved hands.
2. Wipe all materials except the isolate container with ethanol and place in the fume hood, sterilizing the aluminum inoculation loop with a flame.
3. Open isolates under the hood and take a sample of the mycelia using the aluminum inoculation loop while avoiding any agar.
4. Deposit sample into tube* under hood.
5. Be sure to sterilize your tools between different isolates and keep back-up cultures in the incubator.
6. Adjust the temperature of the water bath to 65° C and the centrifuge to 22° C.

7. If samples are fresh, use approximately a 100 mg sample. If samples are lyophilized, use a 20 mg sample.
8. Briefly spin down Rnase A stock solution in the centrifuge to mix condensation with solution.
9. Add 400 µL of buffer AP1 and 4 µl RNase A stock solution (100 mg/mL) and vortex.
10. Incubate the mixture for 10 minutes at 65° C. Mix 2 - 3 times during incubation by inverting the tubes to lyses the cells.
11. Add 130 µL of buffer P3 (formerly called AP2), mix thoroughly.
12. Incubate for 5 minutes in crushed ice or using thermal beads to precipitate any unwanted material remaining in the sample.
13. Centrifuge at 22°C at 14,500 rpm for 5 minutes.
14. Transfer the supernatant to the QIA shredder mini spin column tubes and centrifuge using the 'cold' setting at 14,500 rpm for 2 minutes.
15. Transfer flow-through fraction to a new flip-top tube without disturbing the debris pellet at the bottom.
16. Add 1.5 mL of buffer AW1 and mix immediately by pipetting.
17. Transfer 650 µL of the sample (including any precipitates) to the clear mini spin column (supplied by Qiagen DNeasy Plant Mini Kit). Centrifuge the same at 22°C at 8,000 rpm for 1 minute. Discard the flow-through fraction.
18. Repeat step 17 with the remainder of the sample, in the same mini spin column. Discard the flow-through fraction and the collection tube.
19. Place the mini spin column in a new collection tube (supplied by Qiagen DNeasy Plant Mini Kit) and add 500 µL of buffer AW2 and centrifuge 22°C at 8,000 rpm for 1 minute. Discard flow-through and reuse the collection tube.
20. Add another 500 µl of buffer AW2 and centrifuge 22°Cat 14,500 rpm for 2 minutes. Discard flow-through and the collection tube.
21. Transfer the mini spin column to a 2 mL centrifuge tube (not supplied) and pipette 100 µl of buffer AE onto the DNeasy membrane. Incubate for 5 minutes at room temperature and then centrifuge cold at 8,000 rpm for 5 minutes. Do not discard flow-through fraction.

22. Repeat step 21. The resulting 200 µL contains the DNA.
23. Centrifuge or vortex for 1-2 minutes to collect solution in tube and use the Nanodrop Microvolume Spectrophotometer to estimate concentration of DNA in sample. Use 1 µL of sterile water for a blank test and then test 1 µL of each sample twice.
24. Label all sample tubes clearly with date, sample name and concentration.
25. Store extracted DNA at -20°C (minimum). DNases are active at room temperature and will quickly degrade DNA if not frozen. Samples will last up to 4 years with proper storage.
26. Now, 5 µL of DNA extract solution can be used for PCR reactions.

*Additional notes on desired tubes based on method:

- If lyophilizing, have an empty, sterile 2mL tube ready. Cut out a square section about one cm across and slice away as much agar, which contains contaminating DNA, as possible.
- If using a kit or the boiling method, deposit each sample (either lyophilized or fresh) in 200µL of sterile water in a sterile 2mL tube and homogenize for 20-60 seconds.

APPENDIX B

STANDARD OPERATING PROCEDURE FOR IN-VITRO PROPAGATION OF DWARF WEDGEMUSSEL (*ALASMIDONTA HETERODON*)

Prepared by: _____

Jennifer Ryan Date

Reviewed by: _____

Dr. Allison Roy Date

_____

Dr. David Perkins Date

_____

Timothy Warren Date

_____

Virginia Martell Date

1.0 Scope and Application

1.1. The purpose of this Standard Operating Procedure (SOP) is to describe materials, standard methods, and quality assurance/quality control (QA/QC) measures used in the *in-vitro* propagation of the federally endangered dwarf wedgemussel. Following the steps and methods described in this document will promote consistent techniques for culturing dwarf wedgemussel juveniles using *in-vitro* methods.

1.2. This SOP will be used to outline techniques for dwarf wedgemussel *in-vitro* propagation.

2.0 Method Summary

Methods for *in-vitro* propagation are modeled after Owen *et al.* (2010) and Monte McGregor at the Kentucky Department of Fish and Wildlife Resources Center for Mollusk Conservation who propagated species such

as the elktoe (*Alasmidonta emarginata*), a species in the same genus as the dwarf wedgemussel. All lab and field equipment should be inspected for damage and repaired (as needed) to ensure consistent *in-vitro* culture techniques. All dwarf wedgemussel collections should take place from mid-November to mid-April, when the dwarf wedgemussels are naturally gravid. Only healthy, gravid dwarf wedgemussels that are greater than 26 mm should be collected for broodstock. After dwarf wedgemussels are collected, they should be used for propagation within two months of their collection date. After a mussel is used, it should be returned to its collection site as soon as possible.

In-vitro propagation of dwarf wedgemussel should take place in a solution made up of 67% basal media and 33% serum. Glochidia should take about 14 days to transform (starting on day 0 and transforming on day 13). Proper sterilization techniques should be taken to minimize risk of contamination of the dishes (7.0). After the dwarf wedgemussel have transformed, they should be moved to grow out chambers and divided into three or more chambers.

3.0 **Definitions and Abbreviations**

Coop Unit	The Cooperative Research Unit program in partnership with the United States Geological Survey
Clean bench	Also called a laminar flow hood that circulates filtered air across the work surface to mitigate airborne contamination
.csv	Comma separated values. A transitional file format that be converted from Microsoft Excel to R.
GPS	Global positioning system. Coordinates obtained through GPS are used to mark locations of data loggers on the Earth’s surface
Glochidia	The larval form of unionid freshwater mussels
Gravid	Another word for pregnant when describing a freshwater mussel holding glochidia in her gills

Heat mat	A warming pad generally used for plant propagation, but in this case is used for mussel propagation
Heat treat	A process that involves heating up serum to a temperature of 56°C (or 132°F) for 30 minutes to prevent contamination in culture (Barile 1973).
In-vitro propagation	A method of freshwater mussel propagation where the host fish is replaced by a media solution and glochidia are grown in a culture
PVC	Polyvinyl chloride. Used to make pipes (or lengths of pipes) and cups (in this case, grow out chambers) able to withstand long periods of exposure to water without deteriorating
QA	Quality assurance. The steps taken to ensure that accurate data are collected
QC	Quality control
RO	Reverse Osmosis. A method of water treatment used in conjunction with a calcium reactor and a 0.1-µm filter to create sterile water used in the juvenile grow out system
T2T	Time to transformation. These are the replicates that will be used to determine when the dwarf wedgemussel glochidia are close to transformation and will ultimately indicate when the glochidia are ready for dilution.
USGS	United States Geological Survey

4.0 **Health and Safety Concerns**

Collecting gravid dwarf wedgemussels in the field may pose a hazard associated with working in and over the water. Take precautions such as only going out to collect mussels when flows are low and temperatures are

safely wadeable. All Coop Unit crew members should receive USGS "Over the Water Training", which addresses issues related to working in streams and reservoirs. All Coop Unit members must also receive CPR and First Aid Training. All crew members will wear life jackets in boats and on/near the water, unless diving for mussels. Special certification is needed for using SCUBA. A two-person crew is required while working over water.

Hypodermic needles used in the collection of glochidia from the gravid females pose a rise of poking and can draw blood. Only use hypodermic needles that are enclosed in a sterile wrapper and only remove the needle from its wrapper just before use. In the case where someone accidentally pokes themselves with a needle and draws blood, clean the affected area with an antiseptic cleansing wipe and apply an adhesive bandage.

Ultraviolet (UV) lights are used to sterilize the clean bench prior to making media and working with glochidia. The UV light should be turned off before performing any work under the clean bench, and a sign should be hung up as a reminder. UV light is harmful to personnel and detrimental consequences to glochidia are not known.

Ethanol (70%) will be used for preservation and storage of macroinvertebrate glochidia samples. Ethanol is a Class 1B flammable liquid and requires handling and storage consistent with local fire codes. All Coop Unit and UMass Amherst members must also complete lab and fire safety training at UMass Amherst.

5.0 **Personnel Qualifications**

The person tasked with *in-vitro* propagation of dwarf wedgemussel should have sufficient experience handling freshwater mussels and observing their gills and glochidia. This person must be experienced enough to provide guidance and instruction for less experienced assistants.

6.0 **Dwarf Wedgemussel Collection**

6.1. *Equipment and supplies*

- First aid kit
- Personal floatation devices (PFDs)

- GPS unit
- Pencils and Rite in the Rain (waterproof) paper
- Waders and boots or Wetsuits and snorkels
- Bright, concentrated light
- Small wedge-shaped cork pieces
- Mesh bag to hold collected dwarf wedgemussels
- Hallprint® glue-on shellfish tags (oval, 8 x 4 mm)
- Digital calipers

7.0 **Holding Gravid Dwarf Wedgemussels**

7.1. *Equipment and supplies*

- Full sized Kool-it freezer with shelves and sliding glass doors
- Water pump for an aquarium
- 0.5 m Silicone tubing (2 cm diameter)
- 0.25 m sections of silicone tubing (2 mm diameter)
- PVC pipes with fittings and valves
- Air pump and air stone
- Bio beads
- 6 gallon polyethylene tank
- Tanks equal to the number of gravid females collected
- Reef Nutrition Phyto Feast
- Reed Mariculture *Nannochloropsis* (Nanno) 3600
- Reed Mariculture Shellfish Diet 1800

8.0 **Removing Glochidia from Gravid Mussels**

8.1. *Equipment and Supplies*

- 22G x 1.5 hypodermic needle
- 10 mL syringes (3 per gravid mussel)
- 50 mL syringes (2 per gravid mussel)
- 0.1 µm filters
- 100 mL beakers (at least 3)
- 10 mL simple salt solution
- 2 mL disposable dropper
- Digital laser infrared thermometer
- 0.1 µm sterile filtered water taken from dwarf wedgemussel tanks
- Wash bottles

- Thin pieces of wedged cork
- Bright, concentrated light
- Digital calipers
- 100 mm x 15 mm square gridded petri dish

9.0 **Sterilization**

9.1. *Equipment and Supplies*

- Dawn dish soap
- Kimtech® wipes
- Scotch Brite multi purpose sponges
- 70% ethanol
- UV light
- Nitrile gloves

10.0 **Serum and Basal Media Preparation**

10.1. *Ingredients for basal media (makes approximately 1500 mL of basal media)*

- 10 g M199 powder
- 2.6 g D-(+)- Galactose
- 2.0 g D- (+) - Glucose
- 25 mg 99% L-Ornithine monohydrochloride
- 40 mg L-Taurine
- 0.75 mL MEM Nonessential Amino Acid solution
- 1.5 mL MEM Amino Acid solution
- 200 mg Carbenicillin disodium salt solution
- 200 mg Gentamicin sulfate salt solution
- 200 mg Rifampicin
- 1mg Amphotericin B powder
- 1.5 mL Lipid Mixture
- 1.5 mL MEM Vitamins
- 1.5 mL Menhaden oil
- 1500 mL Chlorine-free water

10.2. *Materials and Supplies*

- 500 mL rabbit serum

- Thermal cooking immersion circulator
- 22 L container with 20L of water
- 2 L Erlenmeyer flask
- 0.1 µm membrane filter with Polyethersulfone
- 0.45 µm membrane filter with cellulose acetate
- 30 mL and 60 mL sterile narrow mouthed plastic bottles - for storing serum
- China marker (black)
- Sodium hydroxide solution
- Hydrochloric acid solution
- 1 mL pipette
- Stirrer plate
- Clean bench with ultraviolet (UV) light
- pH meter
- Gas pressure/vacuum pump with silicone tube sized to fit filter
- Freezer (to at least -30C)
- Refrigerator
- Non-Contact Digital Laser Infrared Thermometer
- Nitrile gloves
- Magnetic spinning bar
- Digital scale
- Plastic weigh boats

11.0 ***In-vitro*** **Culture**

11.1. *Equipment and supplies*

- Stereomicroscope
- China marker
- Disposable plastic or autoclavable glass 60mm x 15mm petri dishes
- 5 mL pipette and pipette tips
- 1 mL pipette and pipette tips
- 200 µL pipette and pipette tips
- Clean bench with UV light
- pH meter
- CO_2 Incubator (Benchmark) model: myTemp Mini CO_2
- Aluminum CO_2 tank
- Heat Mat (brand: vivosun)
- Digital laser infrared thermometer

- 0.1 µm sterile filtered water
- 250 mL squeeze bottles (3)
- Nitrile gloves
- Digital SLR camera
- *In-vitro* dwarf wedgemussel culture data sheet (Appendix A)

12.0 **pH meter**

12.1. *Equipment and Materials*

- Digital pH meter
- pH buffers (4.01, 7.00 and 10.01)
- pH electrode storage solution
- 0.1 µm sterile filtered water in a squeeze bottle

13.0 **Collecting and Holding Gravid Dwarf Wedgemussels**

13.1. *Collection of gravid dwarf wedgemussels*

1. Before collecting gravid female mussels obtain proper federal and state collection permits.
2. Gravid female dwarf wedgemussels can be collected either in the late fall (after mid-November) or the early spring (before mid-April). Depending on the water depth of the site, collecting can either be done by wading and snorkeling, or SCUBA diving. If SCUBA is necessary, use a trained scientist (e.g., from MassWildlife) or contractor (e.g., Biodrawversity, Inc.).
3. While at the site, take a GPS coordinate. This will help place collected dwarf wedgemussels back to their original location.
4. As the mussels are collected, measure their length from anterior to posterior using digital calipers. If the mussel is smaller than 30 mm, then return the mussel to the river. This prevents collecting individuals that are too small or too young.
5. If the mussel is larger than 30 mm, gently pry open the mussel with your fingernails and insert a small piece of wedged cork. Using a bright and concentrated light, look into the mussel and locate the gills. If the gills appear to be inflated and filled with glochidia, then it is gravid. Each mussel should be separately checked by two experts to accurately determine the gravidity of the mussel (Figures B.1 and B.).

a. 

Figure B.1 Example of inflated, gravid gills of a freshwater mussel. Photo courtesy of the Aquatic Epidemiology and Conservation Laboratory at North Carolina State University

b. 

Figure B.2. Inspection of dwarf wedgemussel for inflated gills, indicating gravidity.

6. Collect as many mussels as needed for *in-vitro* work, staying within the confines of the permit. For reference, each gravid dwarf wedgemussel holds about 2500 glochidia (Haag 2013).
7. All mussels should be tagged with 8 x 4 mm oval glue-on shellfish Hallprint® tags with Locktite® super glue gel control.
8. If ready to propagate the mussels *in*-vitro, bring them back to U.S. Fish and Wildlife Service's Richard Cronin Aquatic Resource Center (CARC) in Sunderland, MA in a small, aerated cooler. If you are not yet ready to propagate mussels *in-vitro*, or the glochidia are not fully developed, leave mussels in the river in silos (adapted from Chris Barnhart; Missouri State University) (Figure B.3) until 1 week before propagation.

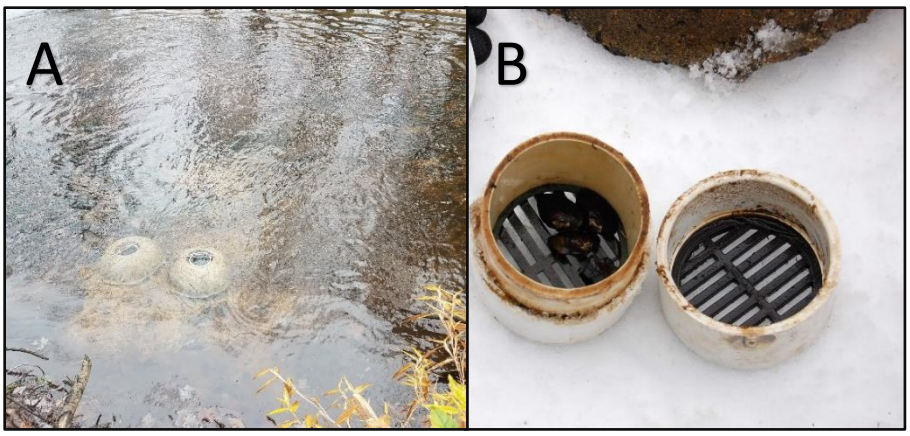
a. 

Figure B.3. In river silos adapted from Christopher Barnhart from Missouri State University. Panel A shows the Silos in River, panel B shows the compartment where the mussels are held within the silos.

9. When the mussels arrive at CARC, follow the Quarantine Standard Operating Procedure written by Ayla Skorupa (2018).
10. If holding mussels for a prolonged period at CARC, store the mussels in a temperature-controlled environment, initially using the same temperature from where they were collected. Bring the temperature to 6°C, only changing the temperature 1–2°C per day to keep the mussels cold enough to prevent the mussels from prematurely releasing their glochidia. If only holding the mussels for 1 week or less, slowly bring the temperature up to 12°C one week leading up to propagation.
11. After the mussels acclimate (about a week), check the mussels' gravidity again. Any mussels found not to be gravid should be returned to their home location

13.2. *Holding Dwarf Wedgemussel Adults Before* In-vitro

1. Keep dwarf wedgemussels in individual containers without any sediment.
2. Every day for 1 week leading up to propagation, do full water changes (explained below in steps a–f) and feed the mussels a mixture of commercially available algae: Reed Mariculture *Nannochloropsis* (Nanno) 3600, Reef Nutrition Phytofeast (Phyto) and Reed Mariculture Shellfish Diet 1800 (Shell).
    a. To do a full water change remove the 5 gallon bucket of filtered, UV treated chlorine-free tap water.

b. Take the clear tube stored in the refrigerator and attach the malleable end to the outflow and put the other end of the pipe into a 22 L container outside the cooler. Open the valve attached to the white tub at the bottom of the cooler so the water pours out.
c. Once the water starts to empty out of the white tub, unplug the cord that is attached to the pump. Immediately close the valve.
d. Empty the 22 L container with the old water.
e. Set the handheld pump in the 5 gallon bucket containing filtered, UV treated chlorine-free tap water and pump the water into the newly emptied white tub.
f. Add 4 mL of Phyto, 0.3 mL of Shell and 0.15 mL of Nanno feed to the white tub using a 1 mL pipette. Mix the feed around in the water thoroughly.
g. Fill the 5 gallon bucket up with chlorine-free tap water and put it back in the cooler so the water will be at the appropriate temperature for the next water change.

14.0 **Keeping a Sterile Environment**

14.1. *Cleaning the Incubator*

1. Two days before planned *in-vitro*, sterilize the inside of the incubator. To sterilize, take the shelves out and wash them with dish soap and very hot water with multi-purpose scrub pads while wearing nitrile gloves. Let the shelves dry, then spray with alcohol.
2. While the trays are drying, spray the inside of the incubator with 70% ethanol and wipe it down with Kimtech® wipes twice.
3. Spray the trays down with 70% ethanol a second time and immediately place them in the incubator.
4. While wearing Nitrile gloves spray down the entire incubator with all the shelving inside and wipe it down. Finally spray one last time with 70% ethanol and close the incubator while it's still wet from ethanol. Turn the incubator on to 23°C with 1.5% CO_2 and let it sit overnight.
5. The day before the planned start date for *in-vitro,* turn on the CO_2 tank and set the PSI to 1 bar (15psi). Mark with tape where the needle lands on the gauge and check it the next day before putting mussels in the incubator to make sure the PSI hasn't changed. If

the needle on the pressure gauge has moved, then something is wrong with the CO_2 tank.

6. Whenever the incubator is opened, unhinge the latch and let it sit for about 2 seconds before slowly opening the door. This step reduces the risk of contaminated air being sucked into the incubator.

14.2. *Keeping a sterile environment under the hood*

1. Before beginning, keep in mind that gloved hands or any materials should not leave the confines of the hood, and if they do, spray with 70% ethanol and wipe with a Kimtech® wipe.
2. Spray again with 70% ethanol and turn on UV light and fan on and leave them on for 10 minutes. Turn the UV light off and leave the fan on before commencing any work under the hood.
3. Before anything is brought under the hood, spray it with 70% ethanol and wipe it down with a Kimtech® wipe. If you are going to open any bottle, then spray the threads of the bottle (the base of the cap) with 70% ethanol and let it sit for a minute before you open it under the hood. Do not breathe on anything and keep your face and bare skin or clothing away from any sterile materials. Always keep hands gloved and remember to work keeping the most contaminated materials to the left of the most sterile equipment.
4. Discard all used materials under the hood including pipette tips and petri dishes.
5. To keep the inside of the plastic spray bottles clean, rinse them with clean hot chlorinated water and then rinse with sterile filtered water. Do not take these spray bottles under the hood; they are not sterile.

14.3. *Sterile Filtering Water*

1. Fill container (100—2000 mL) with unchlorinated water from the tap. Place a magnetic spinning bar inside the container and place on top of a stirrer plate. Turn on the stirrer plate.
2. Get the calibrated pH meter (to see how to calibrate pH meter see 20.1), rinse the probe with sterile filtered water, wipe down with a Kimtech® wipe, and spray with sterile filtered water again. Then place the probe in the stirring water to check the pH.

3. If the pH is lower than 7.65, add sodium hydroxide solution in very small amounts (< 0.5 mL) at a time to raise the pH to 7.65. If the pH is above 7.65, add hydrochloric acid solution in even smaller amounts (< 0.1 mL) at a time to lower the pH to 7.65.
4. After the pH of the water is balanced, filter the water through a 0.1 µm filter. Unscrew the filter and then pour sterile water into a plastic wash bottle.

15.0 **Preparing Media Solution**

15.1. *Preparing Basal Media*

1. Basal media will make up 67% of the media mixture used in *in-vitro* culture.
2. Set a 2 L Erlenmeyer flask with 1500 mL of chlorine free water on a stirrer plate and drop in a magnetic spinning bar.
3. Weigh and measure ingredients (refer to section 10.1 for a complete list of ingredients) on a digital scale in plastic weigh boats (g and mg) or in a pipette (mL) and add to the flask one at a time. The order in which you add your ingredients does not matter
4. Move the 2 L Erlenmeyer flask, the magnetic spinning bar and stirrer plate under the clean bench hood with the UV light turned off.
5. Rinse the calibrated pH probe (20.1) with sterile filtered water and place probe in the stirring basal media to check the pH.
6. If the pH is lower than 7.65, add sodium hydroxide solution in very small amounts (< 0.5 mL) at a time to raise the pH to 7.65. If the pH is above 7.65, add hydrochloric acid solution in even smaller amounts (< 0.1 mL) at a time to lower the pH to 7.65.
7. After the pH of basal media is 7.65, filter the media through a 0.45 µm filter. Put the top on the sterile filtered media and set aside.
8. Then after all of the basal media is filtered down to 0.45 µm, refilter it in 0.1 µm filters.
9. After all of the basal media is filtered down to 0.1 µm, pour media into 30 mL, 50 mL or 60 mL plastic sterile bottles, label with a black china marker and place in a -30°C freezer.

15.2. *Preparing Rabbit Serum*

1. Batches of rabbit serum can be purchased from ThermoFisher Scientific® in quantities of 100 mL or 500 mL. The bottles should be kept frozen at -30°C before use. When you are ready to prepare serum, remove bottles from the freezer and let thaw completely.
2. While waiting for the rabbit serum to warm, prepare a hot water bath with a thermal immersion cooking circulator and a 22L container. Set the temperature on the sous vide to 56°C (or 132°F) (Barile 1973). After the rabbit serum has thawed to room temperature (about 21°C), place the whole bottle in the hot water bath and let sit for 30 minutes. This process will heat treat the rabbit serum to mitigate contamination in culture. Pull rabbit serum out of hot water bath and let it cool back down to room temperature (~21°C).
3. Under the hood, open the rabbit serum and pour it into a 1 L Erlenmeyer flask. Add a magnetic stirring bead, set on top of a stirring plate and place rinsed pH probe (20.1) in the rabbit serum. Get the pH to 7.65 by repeating the same process used for the water and basal media.
4. Filter serum through 250 mL 0.45 µm filters and then 250 mL 0.1 µm filters. The serum should be worked in 50 mL batches to prevent the filter from getting clogged. Divvy up sterile filtered rabbit serum into 30 mL sterile, plastic bottles with 15–30 mL of media in each. Label all the bottles with the amount and type of serum and put in a -30 °C freezer.

16.0 **Extracting Glochidia from Gravid Mussels**

16.1. *Handling Gravid Mussels*

1. Before taking gravid females out of their tanks, scoop out about 500– 1000 mL of the water the mussels are sitting in. Filter this water down to 0.1 µm and set in an ice bath. Keep the water as close to 6°C as possible. Using this water prepare two 50 mL and three 10 mL syringes per mussel. On the 10 mL syringes, screw on a 22 G needle. Keep all of these on ice.
2. Pull out the dwarf wedgemussels and place them in a 100 mL beaker with some water from their tank. When you are ready to

start working with a mussel, remove one from the beaker and gently scrub with a sponge to get the outside of the shell clean. Measure and record the mussels ID, weight, length, and height.

3. Carefully pry open the mussel with your thumbnails and insert a small wedge of cork (< 3 mm thick at its smallest end).
    a. During this process, if your hand slips and the mussel closes, set that mussel aside and work on another individual while the first mussel recuperates.
4. Take both of the 50 mL syringes and gently rinse the inside of the mussel with all 100 mL of water. The inside of the mussel should be as clean as possible before continuing the culture does not become contaminated.
5. Have a second person shine a light into the mussels and look at the gills. Take notes on the gravidity, including: whether the gills on each side are full or partially full and the color of the gills.

16.2. *Glochidia Removal and Volumetric Estimates*

1. To flush the glochidia from the mussel, hold the mussel with one hand and with the other hand slowly stick the 22 G hypodermic needle into the inflated gill of the mussel. Slowly push water from the 10 mL syringe into the gills while holding the mussel over a 100 mL beaker. This pressure will cause the gills to pop and release their glochidia into the beaker. Repeat on the other gill. Thoroughly rinse the inside of the mussel with a 10 mL syringe and 22 G needle into the beaker to get all remaining glochidia.
2. Add sterile water to the beaker so there is 100 mL of water total. Label the beaker with the mussel's Hallprint tag number. Make sure the water is well mixed by gently stirring the water with a pipette tip. Using a 2 mL disposable dropper, place 5, 200 µL drops of glochidia on a gridded petri dish on a 100 mm x 15 mm square gridded petri dish.

3. Using a stereomicroscope with a magnification of 30x, count the number of glochidia within each drop and calculate volumetric estimates for each mussel using the following equation:

$$(Sum\ of\ all\ glochidia\ in\ samples * 200\mu l)$$

4. Repeat this process for each mussel.

5. Throughout this process, allow the temperature of the glochidia to start slowly rise from 12°C to room temperature.

16.3. *Viability Check*

1. The water droplets that were previously used to determine volumetric estimates can now be used separately to determine the viability of each individual mussel. The viability test is modeled after Neves et al. (1985).
2. Percent viability for each mussel should be calculated using the following equation:

$$\left(\frac{Open\ before\ salt\ addition\ -\ Open\ after\ salt\ addition}{Open\ before\ salt\ addition\ +\ Closed\ before\ salt\ addition}\right) * 100$$

3. To calculate volumetric estimates of viable glochidia from each mussel multiply the answers from each equation.
4. Record the viability for each dwarf wedgemussel.

16.4. *Combining Glochidia from All Mussels*

1. Let the glochidia settle to the bottom of the beaker, which should take less than a minute. Decant ¾ of the water off the top of the beaker and pour into a larger beaker in case any glochidia spill out. Repeat for all of the beakers filled with glochidia. Before disposing of the water in the larger beaker, inspect the bottom and spray with a wash bottle to remove any glochidia. Mix all of the beakers with glochidia together into a single beaker. Spray the bottom and sides of all the beakers to ensure that no glochidia are left behind.
2. Pour the glochidia from the beaker into a 100 mm x 15 mm square gridded petri dish and place under a stereomicroscope. With a 1 mL pipette, remove all debris.

17.0 **Starting *In-vitro* Culture**

17.1. *Preparation of the Media*

1. The day before starting the *in-vitro* project, pull out as many basal media and rabbit serum bottles as needed for culture and put the bottles in the refrigerator. Keep in mind that one 60 mL bottle of basal media and one 30 mL bottle of rabbit serum is enough for 18 60 mm x 15 mm dishes with 5 mL (3.35 mL of basal media and 1.65 mL of serum) of media. Pull an additional 30 – 60 mL bottle of basal media that will be used to wash the glochidia.
2. The next day, remove all of the bottles from the refrigerator and let them sit at room temperature when you start pulling glochidia out of the adult mussels.
3. The aim is to get the 6°C media, serum, and glochidia to warm up to room temperature and all be within 1°C of each other when you drop the glochidia into the media solution.
4. While the glochidia are sitting in beakers of sterile water, set out enough 60 mm x 15 mm plastic petri dishes for each replicate.
5. Under the hood, load each of the dishes with 3.35 mL of basal media and 1.65 mL of serum for a total of 5 mL, making sure to keep the lids on the petri dishes as much as possible to minimize exposure to contamination.
6. Set the petri dishes aside with their lids on. Take the additional 30 mL of basal media and pour into a small 50 mL beaker. Set aside.

17.2. *Starting* In-vitro *Culture*

1. Measure the temperature of the glochidia in the petri dish with a non-contact digital laser infrared thermometer. Then check the temperature of the basal media with 30 mL of basal media. These two temperatures should be within 1°C of each other. If the temperatures do not match, place the basal media in the refrigerator or run the serum container under warm water, depending on the need.
2. When the two temperatures match one person should pour the glochidia directly into the beaker filled with basal media, while another person keeps the basal media moving by pumping two 2 mL disposable droppers. Keep moving the media for two minutes.

By keeping the media moving, you minimize the number of glochidia that snap shut on one another.

3. Decant about 20 mL of the basal media and pour the glochidia with the remaining basal media into a sterile 100 mm x 15 mm square gridded petri dish.
4. Measure the temperature of this media and compare to the 60 mm x 15 mm petri dishes with glochidia. These temperatures should also be within 1°C of one another. If not, let all of the petri dishes sit at room temperature until they are the same.
5. Once the temperatures match, count out the desired number of healthy looking glochidia and, with a pipette tip, place them in each 60 mm x 15 mm petri dish. Healthy looking glochidia are glochidia that are not visibly deformed, did not close on other glochidia or remain open when placed in the media.
6. Once all dishes are loaded with glochidia, gently place the dishes in the incubator, stacked in the front of the incubator no more than four high. Do not place any of the petri dishes at the back of the incubator, because you never want to reach in the incubator past the gloved part of your hand.

18.0 **Managing *In-vitro* Petri Dishes**

18.1. *Assessing Contamination*

1. It is likely that the dishes will experience some form of contamination. There are two forms of fungal contamination that have been found in *in-vitro* culture: filamentous and yeast.
2. Yeast can make the dish appear cloudy, and, if not dealt with, can spread ubiquitously throughout the dish. Filamentous fungi, if it forms, usually appears after the yeast, and in stringy, irregular ‘blobs’ that can entangle with glochidia.
3. To assess levels of contamination in the dishes with examples, see Table 2.2.
4. When contamination reaches a score of “2” or higher, it warrants a media change because this is the point where the contamination starts to affect the glochidia’s development negatively.

18.2. *Removal of Non-transformed or Dead Glochidia:*

1. Before performing a media change, check for dead glochidia under a stereomicroscope. Dead glochidia are glochidia that are slightly

open, splayed wide open or are encapsulated in fungus. If you are not performing a media change, do not open the dish to remove the dead glochidia, to prevent possibility of contamination.

2. Once the glochidia start to develop, it is easy to distinguish developing glochidia from non-developing glochidia. A key sign of development is the darkening of the glochidia and then presence of an adductor muscles (Figure B.4.A)
3. On Day 8 or 9 of the experiments, if the adductor muscles in the glochidia are not developing and a glochidia looks transparent instead of dark, then remove those glochidia from the dishes and consider them “dead” or “not transformed” (Figure B.4.B).

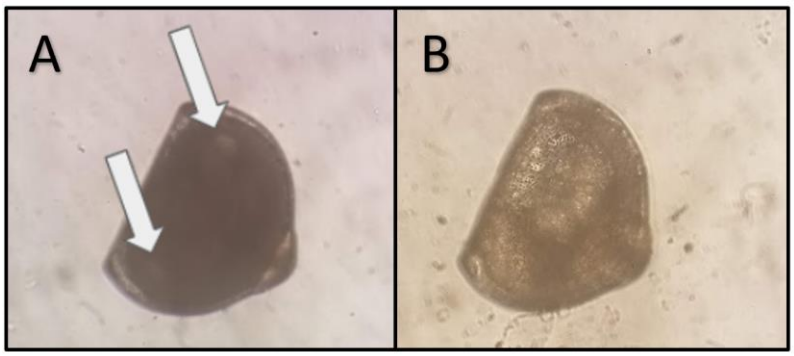
a. 

Figure B.4. Panel A shows a developing glochidia with visible adductor muscles (indicated by white arrows), signifying that the glochidia is close to transformation. Panel B shows a mussel that is dead and will not transform.

18.3. *Media Changes*

1. If there is no contamination in the dish, media changes should be performed as infrequently as possible, as little as 1 or 2 times in a 14 day culture with low densities of glochidia.
2. To supplement media changes, add a 1 mL dose of media (basal and serum) concentrated with Amphotericin B. The dose should be strong enough to bring the dish back up to its original (day 0) concentration of Amphotericin B (1 µg/mL).
3. The day before a planned media change, take out the appropriate amount of basal media and serum out of the freezer and place in the refrigerator to thaw overnight. The morning of a media change, remove both bottles from the refrigerator and let them sit at room temperature. If needed use a sous vide and water bath or a heating mat to warm the serum and media to 23°C.

4. Before starting a media change, look at the dish under a stereomicroscope. Remove and record all open glochidia and any fungal contamination. If there is fungal contamination in the dish, record it (See *Assessing Contamination* (18.1) for more information).
5. Prepare 2 petri dishes of media per media change: the first dish should have 5 mL of basal media and the second dish should have 67% (3.35 mL) basal media and 33% (1.65 mL) serum.
6. Swirl the glochidia to the center of the dish and remove them using a 1 mL pipette. Allow the glochidia to settle to the bottom of the pipette tip and drop the glochidia in a single droplet into the petri dish with only basal media.
7. Look at the glochidia under the microscope again and remove any additional debris, fungus, and open glochidia if you can, adding additional clean basal media to the dish, if needed.
8. Gently swirl the glochidia to the center of the dish, remove glochidia using a pipette and place into the new petri dish with the clean basal media and serum. If there is still some fungus apparent in the dish under the microscope, gently swirl the glochidia for a second time and transfer glochidia for a second time into a new petri dish with basal media and serum.
9. If appropriate, take a picture of the dish using the software AmScope (version 3.7.13522) and then place it back in the incubator. Repeat this process for each dish that requires a media change.

18.4. *Removing Glochidia from the Media*

1. Beginning on Day 10 of the experiment, extract about 10 “test” glochidia from one to two petri dishes
2. The extracted glochidia need to be diluted from the media solution to a freshwater environment.
    a. Remove the test glochidia from their original dish, and move to a holding dish with 5 mL of basal media, set on a heating mat set to 23°C.
    b. Add 1.5 mL of chlorine-free, 23°C, 0.1 µm sterile filtered water with a pH of 7.65 to the holding dish.
    c. After 15 minutes, add 3 mL of the same water to the dish.

d. After 15 additional minutes, add 4 mL of the same water to the dish.
e. Wait 15 minutes.
f. Swirl all of the glochidia to the center of the dish.
g. To remove the remaining media, hold the dish over the sink, tilt the dish slightly, and use a squirt bottle filled with water (as described in step 18.4.2.b), very gently create a whirlpool motion within the dish.
h. Gently squirt the water into the dish, letting the media spill out of the dish slowly until all of the media is removed, and only the water remains.
i. Let the mussels sit for 1-24 hours in the incubator set to 23°C.
j. Check to see how many glochidia are moving 1-24 hours after dilution. On day 10 it is likely that most (if not all) the glochidia won't transform, and will die when diluted. But as you progress to Day 12, the glochidia should move about the dish. Once 50% of the glochidia are moving in the dish after 30 min, incubate the glochidia for another 24 hours and then prepare to dilute all of the glochidia.

3. When the glochidia are ready to be taken out of the media (likely around day 13), turn off the CO_2 on the incubator and prepare a 500–1000 mL of 0.1 µm sterile filtered water with a pH of 7.65 and a temperature of 23°C. Dilute all of the mussels following steps 2a through 2j.
4. One hour after full dilution, look at the dish under a stereomicroscope and record the number of glochidia that are moving (it should be more than half).
    a. Pull out a sample of glochidia from 2 or 3 dishes and put in a petri dish with sterile water. Set these glochidia to the side. These glochidia will be indicators if something is wrong with the grow out system. If the glochidia left in the sterile environment live, and all the other glochidia die, then there is something wrong with the grow out chamber.
5. Hold the transformed juveniles in the incubator set to 23°C with the CO_2 tank off and set to 0.00 on the incubator, until ready to be taken out.
6. Using non-sterile water, bring it up to a temperature of 23°C and add it to the petri dish so there is a mixture of 75% sterile water and 25% nonsterile water. This prepares the glochidia for going into a

non-sterile environment. Make sure when you leave the petri dishes for the night, they are full of water with the lid on.

7. The next day (approximately 24 hours later), check on the glochidia again. Add an additional 25% non-sterile water to the petri dish and this time *do not* change the temperature of the water.
8. After about 10 minutes, move the petri dishes into the growout lab. This lab will be slightly cooler, around 21°C.
9. Transfer the now transformed juveniles to a flow through the system.
10. Unplug and disconnect any flow systems connecting to the holding chambers so none of the glochidia are lost.
11. Float the petri dish on top of the water in the growout chambers. This allows the glochidia to acclimate to the new temperature without abandoning their somewhat sterile petri dish.
12. After a couple of hours, the water in the petri dish should have acclimated to the water in the growout chambers.
13. Gently dump the glochidia into the chambers and use a spray bottle filled with non sterile water to rinse the petri dish so no glochidia get left behind.
14. Repeat steps 3 - 16 for each dish.

19.0 **Data Entry and Management**

19.1. *Data Sheets*

Transcribe handwritten data sheets and extra notes into Microsoft Excel. Enter all data in Microsoft Excel, convert to a .csv file and add files to Rstudio.

19.2. *Counts*

Using pictures taken throughout experiments using the software AmScope (version 3.7.13522), count glochidia using Microsoft Paint. Put a dot on every glochidia counted. Identify closed (alive) and open (dead) from pictures. To see the full procedure for performing glochidia counts see SECTION. When there is a final count, another person should perform a count for the same pictures a second time. For any numbers that don’t match from counts one and two, have a third person count the dishes that did not match.

19.3. *File storage*

Store all the pictures and data files on a flash drive and backup to Google Drive through University of Massachusetts Amherst.

20.0 **Quality Assurance Procedures**

While checking for gravidity of dwarf wedgemussels in the field, two experienced field biologists should always be present to see if the gills are inflated. Both biologists should make the call independently of one another to avoid confirmation bias. Gravidity will be checked a second time in the field to avoid accidentally removing a nongravid individual from the wild unnecessarily.

When working with *in-vitro* culture, pictures of the petri dishes and glochidia should be taken daily. From these pictures you can check contamination, number of open and closed glochidia, any debris in the dish and the color of the dish (which is an indicator of a high or low pH).

All technicians counting glochidia from pictures will first be trained by the lead *in-vitro* culture specialist and follow the format laid out in Appendix B. All pictures will be counted twice by two separate individuals. For any numbers that do not match in counts one and two, a third individual should count the dishes that do not match.

21.0 **Calibrating pH meter**

1. Before calibrating pH meter, the probe to the pH meter should be screwed into a small bottle of Orion electrode storage solution. If it is not, pour some storage solution into the appropriate bottle and let sit for several hours or overnight before attempting to calibrate.
2. On deck, there should be three Orion pH buffers, 4.01, 7.00 and 10.01. Divide each of these three buffers into two 60 mL bottles. Label half of the bottles “buffer” and the other “calibration.” Now you should have six 60 mL bottles in total: “4.01 buffer”, “4.01 calibration”, “7.00 buffer”, “7.00 calibration”, “10.01 buffer” and “10.01 calibration”.
3. To calibrate the pH meter, turn on the pH meter while the probe is still sitting in the storage solution. The pH should read about 4.5. Unscrew the storage solution bottle and rinse the probe with sterile filtered water, wipe with a Kimtech wipe and rinse with sterile

water again. Dip the pH probe into the bottle labeled “4.01 buffer” and then set the probe in the bottle labeled “4.01 calibration.” Hit the “STD” button on the pH meter and wait for the pH meter to read 4.01 (or close to 4.01). Hit the “STD” button again.

4. Repeat this process for the 7.00 solution and then the 10.01 solution in that order. At this point, pH meter should be calibrated.
5. This process should be repeated on a weekly basis to ensure accurate readings.

22.0 **Waste Disposal**

Prior to disposal, unused ethanol must be collected and diluted to a concentration of less than 23%.

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